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Research Paper

A novel enzyme-assisted one-pot method for the extraction of fucoidan and alginate oligosaccharides from *Lessonia trabeculata* and their bioactivities*

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Abstract Alginate oligosaccharides (AOS) and fucoidan are two important bioactive carbohydrate which that can be prepared from brown seaweeds. We investigated a novel enzyme-assisted one-pot method on AOS and fucoidan extraction from *Lessonia trabeculata*, and found that the optimum extraction procedure was: first, using 10-g/L Na_2CO_3 solution to digest the seaweeds for 2 h at 80 °C with the material-liquid ratio 1∶20 (g/mL), and then adding 4.0% fucoidan cleavage enzyme for reaction for 12 h at 45 °C. The yield of AOS and fucoidan was 21.36% and 14.40%, respectively, which was higher than those using traditional methods. The degree of polymerization of AOS was determined to be 3–5, confirming the results through thin layer chromatography (TLC) and electrospray ionization mass spectrometry (ESI-MS). The ratio of guluronic acid to mannuronic acid in AOS was found to be 1.97. Through anion-exchange column chromatography, four sulfated polysaccharide fractions (F0.5, F1.0, F1.5, and F2.0) were successfully isolated from fucoidan. Chemical analysis revealed that fucoidan and F0.5 were heteropolysaccharides, consisting mainly of mannuronic acid, fucose, and xylose, while the other fractions were predominantly composed of fucose and galactose. The sulfate group content increased from 8.74% to 18.76%, while the uronic acid content decreased from 23.79% to 5.58% across the fractions. All fractions of fucoidan exhibited significant antioxidant activity in various tests. There was a positive correlation between the sulfate content and the ability to scavenge superoxide radicals. Additionally, both AOS and the fucoidan fractions showed immunostimulation activity and were non-toxic to RAW264.7 cells. These results indicate that the novel enzyme-assisted one-pot method is a green and efficient approach for the extraction of fucoidan and alginate oligosaccharides from brown seaweeds. It has potential applications in the processing industry of brown seaweeds.

Keyword: *Lessonia trabeculata*; alkaline digestion-enzymatic hydrolysis; alginate oligosaccharides; fucoidan; antioxidant activity

1 INTRODUCTION

Alginate and fucoidan are two important natural anion polysaccharides extracted from the brown seaweeds. Alginate, also known as kelp gum, or seaweed gum, is the predominant polysaccharide in the cell walls and intercellular matrix of brown

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algae. Alginate was initially produced in the United Kingdom, and then large scale produced in the United States and France (Al Monla et al., 2022). Besides marine brown algae, alginate can also be synthesized by microorganisms. Alginate is a hydrophilic colloid that possesses excellent thickening, gelation, biocompatibility, and non-toxic properties (Vasudevan et al., 2021). These characteristics have led to its wide utilization in food, medicine, and biological materials. However, alginate's high molecular weight, viscosity, and limited bioavailability have hindered its further application and development. Alginate oligosaccharides (AOS), low molecular weight polymers derived from alginate, have gained increasing research interest due to their lower viscosity, better water solubility, easy absorption, enhanced bioactivity, and good stability (Xing et al., 2020). Consequently, the study of AOS has been on the rise (Zhang et al., 2020).

AOS are functional oligomers with a degree of polymerization (DP) ranging from 2 to 20, and their molecular weights typically range from 200 to 4000 Da. They are composed of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Urtuvia et al., 2017). Alginate degradation is the primary method used to prepare AOS. Currently, there are three main degradation methods for alginate: physical degradation, chemical degradation, and biodegradation. Physical degradation methods include ultrasonic and supercritical degradation, as well as radiation. However, the efficiency of ultrasonic degradation using ethanol as a solvent decreases significantly as the concentration of alginate increases, and it also prevents the gelation of alginate (Wardhani et al., 2021). Supercritical degradation primarily utilizes supercritical water or subcritical water. Supercritical water can completely degrade alginate, but it requires high temperatures and pressures. Subcritical water selectively hydrolyzes M-G and G-M glycosidic bonds (Vasudevan et al., 2021). Radiation degradation efficiency also decreases with increasing alginate concentration (Wasikiewicz et al., 2005). Chemical degradation methods include oxidative degradation, acid degradation, and alkaline degradation. Oxidative degradation mainly uses oxidants to break the glycosidic bond, but it often results in the oxidation of the aldehyde group at the C-1 position to a carboxylic acid group (Yang et al., 2004). Acid degradation and alkali degradation methods tend to break down the structure of alginate (Niemelä and Sjöström, 1985), so these two methods are not commonly used. Chemical degradation methods also has long reaction time (Zimoch-Korzycka et al., 2021), difficult control of reaction conditions (Lu et al., 2009), and can contribute to environment pollution. In contrast, biodegradation methods, such as enzymatic degradation, are more suitable for obtaining AOS. Biodegradation methods offer advantages such as high catalytic efficiency, excellent substrate specificity, mild reaction conditions, energy-saving, and environmental friendliness (Zhu et al., 2021). Moreover, while physical and chemical degradation methods are non-specific, AOS obtained through microbial degradation have a C=C bond at the end, which may enhance their bioactivities (Vasudevan et al., 2021; Zimoch-Korzycka et al., 2021). Therefore, the study of enzymatic methods for the preparation of AOS has gained significant attention in recent years. Currently, to obtain AOS, alginate is first extracted from seaweeds and then degraded to AOS. This two-step extraction method is complex, and fucoidan, which is also present in seaweeds, often remains in the extraction solution or seaweed residue and goes to waste. If there were a method to directly extract AOS from seaweeds, it would not only reduce the excessive use of chemical reagents in alginate extraction but also improve production efficiency.

Fucoidan is a water-soluble sulfated heteropolysaccharide found in various algal and echinoderms. It processes multiple biological activities, including antioxidant, anti-tumor, anti-inflammatory, immunomodulatory and anti-cardiovascular functions. The bioactivities of fucoidan are generally depended on factors such as sulfate content, monosaccharide composition, molecular weight and the linkage of the monosaccharide (Cunha and Grenha, 2016). There are several main methods for extracting fucoidan from brown algae, including hot water extraction, alkali extraction, microwave-assisted extraction, and enzyme extraction. Hot water extraction is environmentally friendly, economical, convenient, and preserves the natural structure of polysaccharides. However, it is challenging to break the cell wall, resulting in a low extraction rate of polysaccharides (Acosta-Estrada et al., 2014). Alkali extraction can yield a high amount of polysaccharides (Ale et al., 2011), but strict pH control is necessary to prevent fucoidan degradation (Morya et al., 2012). Microwave-assisted extraction

is easy to operate, provides high polysaccharides yield, and has a short extraction time. However, it consumes a significant amount of power, may damage polysaccharides activity, and is not suitable for large-scale extraction (Liu et al., 2018). Compared to the aforementioned methods, enzyme-assisted extraction of polysaccharides is more efficient (Chen et al., 2020) and preserves biological activity, thus offering broader application prospects (Abuduwaili et al., 2019). Currently, obtaining fucoidan and AOS typically involves three steps. Firstly, fucoidan is extracted from seaweeds. Secondly, alginate is extracted from seaweeds. Finally, alginate is degraded to obtain AOS. If fucoidan and AOS could be directly extracted from seaweeds, seaweed resources could be maximally utilized, and the extraction efficiency of polysaccharides could be greatly improved.

Lessonia trabeculata, a species belonging to the Phaeophyta, Laminariales, Lessoniaceae, *Lessonia*, grows in the subtidal zone of the shallow coastal area along the Pacific coast of South America (Murúa et al., 2013). Substantial amounts of *Macrocystis* (*M. pyrifera*) and *Lessonia* (*L. nigrescens* and *L. trabeculata*) are traditionally harvested and exported from Chile and South Africa as raw material for alginate production (Westermeier et al., 2006). However, the extraction conditions for alginate from *L. trabeculata* are more challenging compared to other brown algae species. For example, it requires a higher concentration of Na_2CO_3, higher temperature, and longer extraction time. The cell wall of *L. trabeculata* consists of various polysaccharides, including alginate, fucoidan, cellulose, hemicellulose, etc., which contributes to the lower extraction rate of alginate (Togashi et al., 2009).

Therefore, in this study, a novel enzyme-assisted one-pot method was established to extracted fucoidan and AOS from *L. trabeculata*. This method combined alkaline digestion and enzyme hydrolysis to effectively break down the complex cell wall structure and obtain fucoidan and AOS simultaneously. The conditions of the enzyme-assisted one-pot method were optimized using a one-factor control approach. Subsequently, the crude AOS and fucoidan were purified and subjected to chemical property analysis. Finally, the immunostimulation activity and in vitro antioxidant activity of the extracted compounds were evaluated.

2 MATERIAL AND METHOD

2.1 Material

Lessonia trabeculata was collected along Pacific coast near Chile in 2022. The fresh seaweed was soon washed and dried, and crushed into size powders in 1 000 mesh.

2.2 Chemical

Phenol, anhydrous sodium carbonate, anhydrous sodium sulfate, concentrated sulfuric acid, silver nitrate, n-butanol, formic acid, DMSO, ferric chloride, potassium ferricyanide, trichloroacetic acid, all are analytically pure, purchased from Sinopharm Chemical Reagent Co. Sodium carbonate, sodium bicarbonate are ion chromatography grade, methanol and acetonitrile were chromatographically pure, purchased from Merck KGaA, Germany. Dextrose series standard, chromatographically pure, purchased from China Institute for the Control of Pharmaceutical and Biological Products. Guluronic acid, purchased from Shandong Qingdao Bozhihuili Biotechnology Co., Ltd. Mannuronic acid standard, purified by our laboratory. Alginate cleavage complex enzyme sourced from *Bacillus subtilis* (CCTCC No: M2020698) was purchased from Weifang Kandion Co. Sodium alginate, purchased from Qingdao Mingyue Seaweed Group Co.

2.3 Enzyme assisted one-pot method

In this experiment, the extraction conditions of alkaline digestion combined with complex enzyme hydrolysis method were investigated using a one-factor control method. The optimal conditions were determined based on the maximum dissolution rate of water-soluble substances. Firstly, the effects of material-liquid ratio (1∶12, 1∶16, 1∶20, 1∶24, 1∶28, w/v), alkaline digestion concentration (10 g/L, 15 g/L, 20 g/L), alkaline digestion temperature (70 °C, 80 °C, 90 °C), and alkaline digestion time (2 h, 4 h, and 6 h) on the dissolution of water-soluble substances were studied. Secondly, the addition amount of alginate cleavage complex enzyme (2.0%, 2.0%, 6.0%, g/g), enzyme digestion time (6 h, 12 h, 18 h) were controlled to investigate the effect on the dissolution of water-soluble substances. After determining the optimal process conditions, 50-g *L. trabeculata* algal powder was used for the extraction of AOS and fucoidan.

The detail of the enzyme assisted one-pot method

was as following. According to a certain material-liquid ratio, a certain concentration of Na_2CO_3 was added and digested. The pH was adjusted to 6.8±0.2 with KH_2PO_4, and a certain amount of alginate cleavage complex enzyme was added, and enzymatic hydrolysis was carried out at 45 °C. After centrifugation, the supernatant was inactivated at 95 °C for 15 min, and the solution was centrifuged to remove the precipitate. $CaCl_2$ solution was added to the supernatant to a final concentration 0.5 mol/L. The supernatant was dialyzed with a 12-kDa dialysis bag. The exudate and retention solution were collected separately, concentrated and lyophilized to obtain crude AOS and fucoidan. The flow chart of the extraction process is shown in Fig.1.

2.4 Purification of AOS and fucoidan

2.4.1 Purification of AOS

The crude AOS is desalted using a multifunctional membrane separation plant (LNG-UF-101, Shanghai Langji Membrane Separation Equipment Engineering Co.). The ultrafilter was circulated with distilled water until the conductivity of the effluent and distilled water were nearly the same. In this experiment, the filter membrane was selected to retain the molecular weight of 160 Da (S-NF160, Shanghai Langji Membrane Separation Equipment Engineering Co.).

AOS was further purified using Bio-Gel P-4 Gel (2.6 cm×100 cm). The column was eluted with 0.5-mol/L NH_4HCO_3 solution at a flow rate of 16.8 mL/h. The eluate (5.6 mL/tube) was collected automatically (SBS-100, Shanghai Husi Analytical Instrument Factory Co.). Polysaccharide fractions were analyzed using the phenol-sulfuric acid method (Dubois et al., 1956).

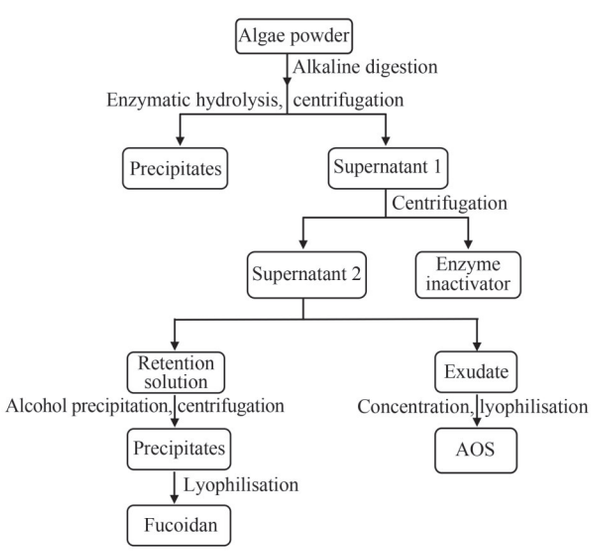

Fig.1 Enzyme assisted one-pot methods flow chart of AOS and fucoidan from *L. trabeculata*

2.4.2 Purification of fucoidan

Fucoidan further fractionation was performed using anion-exchange chromatography. Fucoidan was dissolved in H_2O and applied to DEAE-Sepharose fast flow column (2.6 cm×60 cm, Amersham Biosciences). Fractions were prepared in stepwise elution with increased concentration of NaCl (0.5, 1.0, 1.5 and 2.0 mol/L) solution in turn at a flow rate of 3 mL/min. The elution was detected by phenol-sulfuric acid method (Bradford, 1976). Each elution was combined, dialyzed, concentrated in a rotary evaporator, and finally lyophilized in a freeze dryer (Tokyo Rikakikai Co., Ltd., Japan).

2.5 Chemical property of fucoidan and AOS

2.5.1 Chemical analysis

Protein content was measured in the method of Bradford (1976). Total sugar and fucose content of each fraction was determined according to the method of Dubois et al. (1956) and Grice (1988) using fucose as standard (50 and 10 mg/mL). Uronic acid was estimated in a modified carbazole method using D-glucuronic acid as standard (Bitter and Muir, 1962).

2.5.2 Determination of molecular weight (Mw)

Mw of samples was determined using high-performance gel permeation chromatography (HPGPC) described by Guo et al. (2018). To determine the Mw, a set of standard substances of series dextran were used, including T2700, T5250, T9750, T500, T13050, T36800, T64650, and T135350. The HPGPC system employed TSK G3000 PWxl gel column and a refractive index detector (Agilent, USA). The retention time (t_R) of the chromatogram for each polysaccharide standard sample was determined. The logarithm of the molecular weight of the polysaccharide standard (logMw) was then plotted against the t_R to generate a standard curve.

2.5.3 Degree of polymerization (DP) of AOS

Thin layer chromatography (Chen et al., 2018) was used to initially determine the DP of AOS. TLC-separation was performed on commercially available silica gel. N-butanol/formic acid/water (6∶4∶1,

v/v/v) were used as the developing solvent and alginate oligosaccharide standard (DP: 1–5, without DP=2) (5 mg/mL) were applied. The silica gel plates were visualized by heating at 105 °C for 10 min after sprayed with 5% sulfuric acid-ethanol solvent. Finally, ESI-MS was used to further validate the DP of AOS.

2.5.4 FT-IR spectroscopy analysis

Infrared spectra of polysaccharide were measured in the range of 4 000–400/cm at room temperature by using Fourier transform infrared spectrophotometer (Thermo Fisher, Waltham, MA, USA) (Geng et al., 2018a).

2.5.5 Analysis of monosaccharide composition

The monosaccharide composition of the samples was determined by high performance liquid chromatography with PMP-pre-column derivatives according to Geng et al. (2018b). Chromatographic conditions were generally as follows: ORBAX SB-Aq column, 5 μm, 4.6 mm×250 mm; temperature, 25 °C; Acetonitrile: PBS (0.1 mol/L, 6.8) =83∶17 at 0.8 mL/min. The eluate was monitored at 245 nm. Guluronic acid and mannuronic acid were used as standards to measure the ratio of G/M in purified AOS. Glucose (Glc), Rhamnose (Rha), Glucuronic acid (GlcA), Fucose (Fuc), Xylose (Xyl), Mmannose (Man), Galactose (Gal), and Mannose acid (ManA) were used as standards for the determination of the monosaccharide composition of fucoidan and fractions.

2.6 Macrophage proliferation activity and nitric oxide releasing capacity

100 μL of RAW264.7 cells were seeded (1×10^4 cells/well) in 96-well plates and incubated for 24 h at 37 °C with 5% CO_2. For the cell viability assay, 100 μL of polysaccharide samples (25, 50, 100, 200 μg/mL) and lipopolysaccharide (LPS, 1 mg/mL) were added to each well. The plate was then incubated for 24 h at 37 °C with at least three replicate wells per set. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following the method described by Mosmann (1983). MTT solutions were prepared in PBS (pH= 7.4), filtered (0.22 μm) prior to use, and stored in the dark at 4 °C for no more than two weeks. To perform the MTT assay, 10 μL of MTT solution was added to each well and the plate was incubated at 37 °C for 4 h. The supernatant was then discarded, and 100 μL of DMSO was added to dissolve the purple formazan crystals. The plate was shaken well at room temperature for 10 min, and the absorbance of the samples was measured at 490 nm using a microplate reader. For the quantification of nitric oxide (NO) production in macrophage cells, 100 μL of RAW264.7 cells were seeded in a 96-well plate as described above and incubated at 37 °C for 24 h. The cells were treated with different concentrations of polysaccharides (25, 50, 100, 200 μg/mL) and lipopolysaccharide (LPS, 1 mg/mL). After incubation at 37 °C for 24 h, 50 μL of the supernatant was taken and mixed with Griess reaction solution. The mixture was thoroughly mixed for 10 min at room temperature, and the absorbance of the samples was measured at 540 nm using a microplate reader. The quantification of nitric oxide (NO) production was determined by comparing the absorbance values with a sodium nitrite standard curve, following the method described by Green et al. (1982).

2.7 Antioxidant activity assay

2.7.1 DPPH free radical scavenging activity

The scavenging activity of 1,1-diphenyl-2-picryl-hydrazyl radical refers to the method in the literature with slight modifications (Wang et al., 2010). A series of sample solutions with different concentrations (0.25, 0.50, 1.0, 2.0, 4.0, 8.0 mg/mL) were prepared using deionized water. To perform the assay, 1 mL of each sample solution with different concentrations was mixed with 2 mL of a 7.1-μg/mL DPPH anhydrous ethanol solution. The mixture was vigorously shaken and allowed to stand in the dark at room temperature for 30 min. The absorbance of the reaction mixture was then measured at 517 nm. An anhydrous ethanol solution was used as a control group instead of the sample. The scavenging activity of DPPH free radicals was calculated using the following formula:

$$\text{Scavenging effect (\%)}=(1-OD_{sample}/OD_{control})\times100\%.$$

2.7.2 Determination of the reducing power

The reducing power was tested with reference to the method in the literature with slight modifications (Wang et al., 2010). A series of sample solutions of different concentrations (0.25, 0.50, 1.0, 2.0, 4.0, 8.0 mg/mL) were prepared with PBS (0.2 mol/L, pH=6.6). To carry out the assay, 0.13 mL of each sample concentration was mixed with 1.25 mL of $K_3[Fe(CN)_6]$ (1%, w/v) and incubate at 50 °C for

20 min. The reaction was terminated by adding 1.25 mL of trichloroacetic acid (TCA, 10%, w/v) to the reaction system. Subsequently, 1.5 mL of $FeCl_3$ solution was added, and the absorbance of the resulting solution was measured at 700 nm.

2.8 Statistical analysis

Each experiment was conducted in triplicate to ensure the reliability of the results. Statistical analysis was performed by the Statistical Analysis Systems (GraphPad Prism 8.0.2).

3 RESULT

3.1 Factor analysis of enzyme assisted one-pot extraction methods

The dissolution of water-soluble substances in *L. trabeculata* can be assessed by measuring the yield of algae residues. A high dissolution of water-soluble substances corresponds to a low yield of algae residues. The solid-liquid ratio was found to have an impact on the yield of algae residues. When the solid-liquid ratio ranged from 1∶12 to 1∶28, the yield of algae residues decreased, with the lowest yield observed at a ratio of 1∶20 (Fig.2). The concentration of alkali, digestion temperature, and digestion time were found to significantly affect the dissolution of water-soluble substances (Fig.3). Higher alkali concentration and longer digestion time resulted in lower levels of water-soluble substances. Additionally, digestion at 80 °C yielded the highest amount of water-soluble substances. The amount of enzyme and hydrolysis time had a relatively smaller impact on the yield of algae residues. When 4.0% enzyme was added, the yield of algae residues was 26.47%. Meanwhile, when the enzyme addition amount was 6%, the yield of algae residues was 24.16% (Fig.4). Considering the economic benefits of enzyme usage, an enzyme addition amount of 4.0% was chosen.

Based on the results of the single-factor experiments, the optimal process conditions are summarized in Table 1. To confirm these optimal conditions, a confirmatory experiment was conducted using 50 g of algae powder. The yields of crude AOS and fucoidan were found to be 87.19% and 14.4%, respectively. Additionally, the yield of

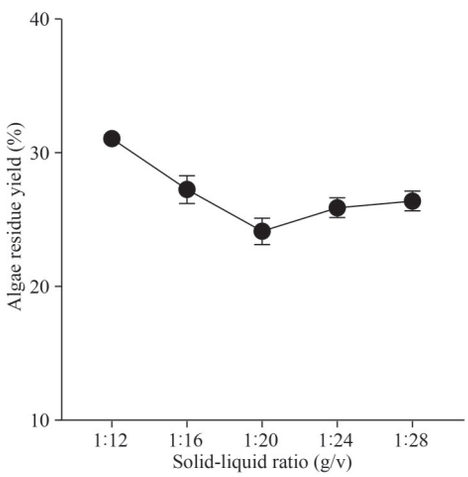

Fig.2 The effects of solid-liquid ratio on the yield of algae residues

10-g/L Na_2CO_3 was added and digested at 80 °C for 2 h, 6% alginate cleavage complex enzyme was added and digested at 45 °C for 18 h.

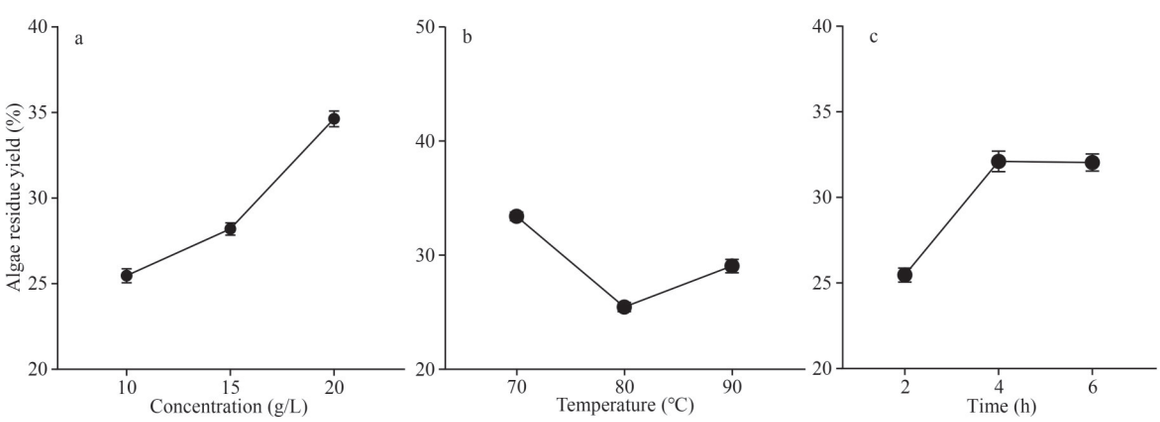

Fig.3 The effects of alkali digestion concentration (a), temperature (b), and time (c) on the yield of algae residues

a. a solid-liquid ratio was 1∶20, and the concentration of Na_2CO_3 was investigated. The digestion was carried out at 80 °C for 2 h, and 6% alginate cleavage complex enzyme was added and digested at 45 °C for 18 h; b. a solid-liquid ratio was 1∶20, and the concentration of Na_2CO_3 was 10 g/L. The digestion was carried out at different digestion temperature for 2 h, and 6% alginate cleavage complex enzyme was added and digested at 45 °C for 18 h; c. a solid-liquid ratio was 1∶20, and the concentration of Na_2CO_3 was 10 g/L. The digestion was carried out at 80 °C for different time, and 6% alginate cleavage complex enzyme was added and digested at 45 °C for 18 h.

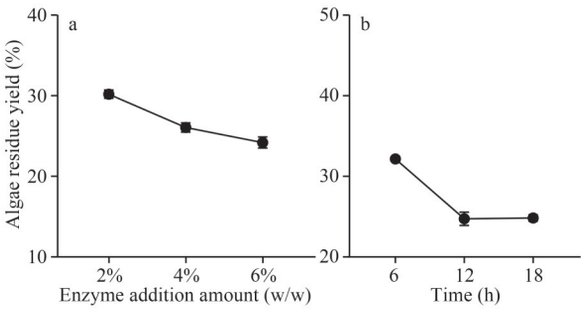

Fig.4 The effects of the amount of enzyme (a) and hydrolysis time (b) on the yield of algae residues

a. a solid-liquid ratio was 1:20, and the concentration of Na_2CO_3 was 10 g/L. The digestion was carried out at 80 °C for 2 h, then alginate cleavage complex enzyme was added and digested at 45 °C for 18 h; b. a solid-liquid ratio was 1:20, and the concentration of Na_2CO_3 was 10 g/L. The digestion was carried out at 80 °C for 2 h, then 6% alginate cleavage complex enzyme was added and digested at 45 °C.

alginate was 0.19%. These results demonstrate that the established method effectively extracts AOS and fucoidan from the algae.

3.2 Purification and chemical analysis of AOS

AOS was obtained after the purification of crude alginate oligosaccharides in a multifunctional membrane separation plant (LNG-UF-101, Shanghai Longyi Membrane Separation Equipment Engineering Co.). The yield of AOS from *L. trabeculata* was about 21.36%. To analyze the composition of AOS, each tube with a high sugar content was tested using TLC. The TLC results revealed that AOS mainly consisted of trisaccharides, tetrasaccharides, and pentasaccharides (Fig.5). To further determine the degree of oligosaccharide polymerization, the AOS was subjected to ESI-MS analysis. The negative-ion mode ESI-MS of AOS indicated a relatively simple composition. The spectra showed that the fragments of singly charged ions were observed at *m*/*z* 527.09 and 703.12, corresponding to trisaccharides and tetrasaccharides, respectively. The fragments of doubly charged ions were observed at *m*/*z* 263.04, 351.06, and 439.07, corresponding to trisaccharides, tetrasaccharides, and pentasaccharides of the glyoxalate, respectively (Fig.6).

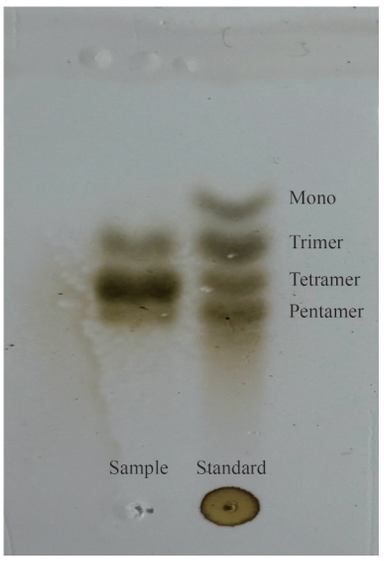

Fig.5 TLC analysis of the fractions

Saturated alginate oligosaccharide standard (DP: monosaccharide, 3–5).

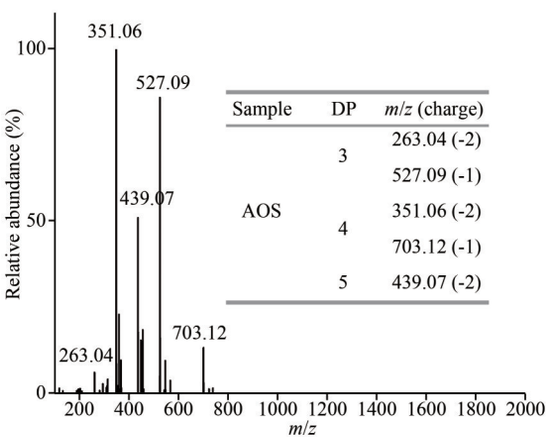

Fig.6 ESI-MS analysis of the AOS

AOS is a low molecular polymer composed of guluronic acid (G) and mannuronic acid (M) monomers linked by glycosidic bonding. The efficacy of AOS can vary depending on the composition of uronic acids present. High-performance liquid chromatography (HPLC) analysis, as shown in Fig.7, was performed to determine the ratio of G/M in the AOS sample. The measurement revealed a G/M ratio of 1.97, indicating that this particular sample contains a higher proportion of glucuronic acid.

Table 1 Effect of factors on the dissolution of water-soluble substances in *Lessonia trabeculata*

	Material-liquid ratio (w/v)	Alkali concentration (g/L)	Digestion temperature (°C)	Digestion temperature time (h)	Enzyme addition (%)	Enzyme digestion time (h)
	1:20	10	80	2	4.0	12
Algae residue yield (%)	23.52	25.12	25.00	25.68	26.47	23.72

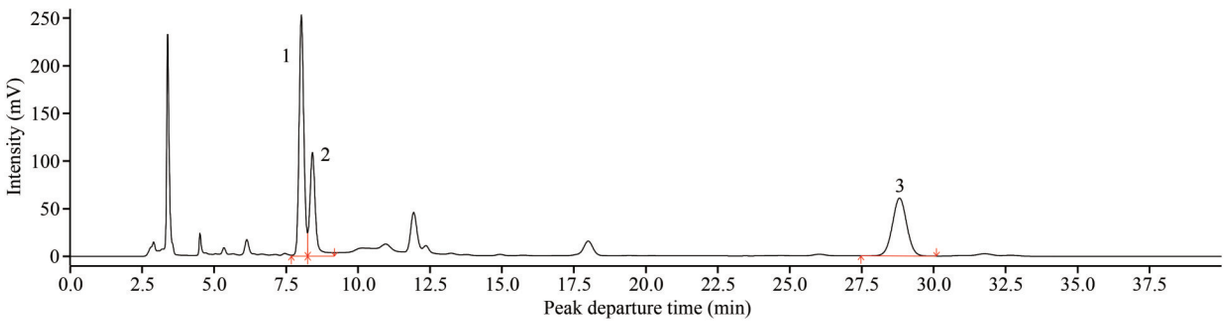

Fig.7 High performance liquid chromatography of algin oligosaccharides

1: guluronic acid; 2: mannuronic acid; 3: ribose.

3.3 Purification and chemical analysis of fucoidan

Fucoidan was separated into four fractions, namely F0.5, F1.0, F1.5, and F2.0, using different concentrations of NaCl solution. The yields of these fractions were 41.76%, 8.70%, 1.41%, and 0.89%, respectively. The chemical composition of each fraction is presented in Table 2. The overall yield of fucoidan was determined to be 14.4%, with a sulfate content of 12.53%. The total sugar content was found to be 83.39%, with fucose, uronic acid, and protein contents of 15.30%, 27.48%, and 12.93%, respectively. Among the fractions, F1.0 and F1.5 had the highest total sugar content, while F2.0 had the lowest. With increasing ionic strength of the eluant, the amount of uronic acids decreased, while the content of sulfate groups increased. All samples contained sulfate groups, with contents ranging from 8.74% to 18.76%. Additionally, proteins were present in all fractions, with the lowest protein content observed in the F1.0 fraction. In terms of sugar composition, mannuronic acid and fucose were the main sugar units in fucoidan and F0.5, with very low content of other sugar units except xylose. Fucose was the major unit in F1.0, F1.5, and F2.0, followed by glucose and xylose. The molecular weight analysis revealed that F0.5 had the lowest molecular weight, while F2.0 had the highest. The remaining fractions had molecular weights ranging from 110–214 kDa.

3.4 FT-IR spectrum analysis

To characterize the structural properties of fucoidan, we utilized FT-IR spectra in the wavenumber range of 400–4 000/cm, specifically focusing on the polysaccharide fingerprint region (Fig.8). The strong broadband observed at 3 284.55/cm indicates the presence of O-H group stretching, which is commonly observed in polysaccharides (Mohamed et al., 2012). The peak at 2 928.05/cm is attributed to the C-H stretching vibration (Ganapathy et al., 2019). The absorption peaks around 1 600/cm and 1 400/cm represent the

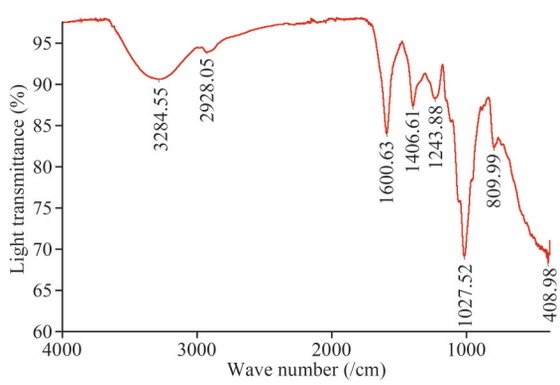

Fig.8 Infrared spectrum of fucoidan

Table 2 Analysis of the physical and chemical properties of fucoidan and each fractionated component

Fraction	Yield (%)	Protein content (%)	Total sugar (%)	Fucose (%)	Uronic acid (%)	Sulfate (%)	Avg. Mw (kDa)	Monsaccharide composition, molar ratio							
								ManA	Man	Rha	Gal	GlcA	Glc	Xyl	Fuc
Fucoidan	14.40	12.93	83.39	15.30	27.48	12.53	122.3	1.00	0.17	0.02	0.11	0.40	0.13	0.58	0.87
F0.5	41.76	7.94	78.03	18.96	23.79	8.74	110.2	1.00	0.16	nd	0.11	0.20	nd	0.70	0.96
F1.0	8.70	1.14	93.91	21.69	12.36	10.31	139.1	0.10	0.17	0.10	0.09	nd	0.93	0.36	1.00
F1.5	1.41	8.06	80.54	29.44	9.70	14.54	116.5	nd	0.03	0.10	0.03	0.02	0.73	0.11	1.00
F2.0	0.89	5.37	31.91	8.03	5.58	18.76	214.2	nd	0.08	0.02	0.02	0.03	0.32	0.10	1.00

“nd” means not detected.

stretching vibration of C=O in glyoxylate and C-O in sugar ring, respectively (Razmkhah et al., 2016; Wang et al., 2017). The absorption peaks in the range of 800–1 243.88/cm correspond to the stretching vibration of S=O in the sulfate group. This characteristic absorption peak indicates that the polysaccharide is a sulfated polysaccharide (Nagahawatta et al., 2022). Futthermore, peak absorption peaks around 1 000/cm confirms the presence of glyoxalate (Nep and Conway, 2011; Wang et al., 2013).

3.5 Immunostimulation activity

Due to the low yield of F1.5 and F2.0 fractions, their biological activity evaluation was not possible. Therefore, only the F0.5 and F1.0 fractions were used for bioactivity studies. RAW264.7 cells were exposed to AOS, fucoidan, and its fractions at concentrations of 25, 50, 100, and 200 μg/mL for 24 h to assess cytotoxicity. As depicted in Fig.9a, all tested samples exhibited no cytotoxic effects on the RAW264.7 cells. Furthermore, the production of nitric oxide (NO) was significantly increased in a dose-dependent manner by the treatment of each sample compared to the blank group (Fig.9b). The order of NO production decrease was as follows: fucoidan, F1.0, F0.5. Notably, the immunostimulatory activity of F1.0 was significantly higher than that of fucoidan at a concentration of 200 μg/mL. The concentration of NO induced by LPS (1 μg/mL) was 34 μmol/L, while the concentrations of NO induced by fucoidan (200 μg/mL), F1.0 (200 μg/mL), and AOS (100 μg/mL) were 34 μmol/L, 41 μmol/L, and 34.5 μmol/L, respectively.

3.6 Antioxidant activity assay

3.6.1 DPPH radical scavenging activity

The antioxidant activity of polysaccharides is commonly evaluated by their ability to scavenge DPPH radicals, which provides a quick assessment of their antioxidant potential. The stronger the sample's ability to scavenge DPPH, the higher its antioxidant activity. The concentration-dependent DPPH radical scavenging capacities of each component are shown in Fig.10. The IC_{50} values of fucoidan, F0.5, F1.0, and AOS could not be determined. However, as the concentration increased from 0.25 mg/mL to 8 mg/mL, the scavenging capacity of AOS increased from 18.92% to 36.44%, while that of F0.5 increased from 19.83% to 38.30%. The scavenging capacities of AOS and F0.5 for DPPH radicals were almost the same. The scavenging capacities of fucoidan and F1.0 were consistent between 0–2 mg/mL. However, between 2–8 mg/mL, the scavenging ability of F1.0 was significantly better than that of fucoidan.

3.6.2 Determination of the reducing power

The determination of reducing power is based on the generation of prussian blue $Fe_4[Fe(CN)_6]$, where the antioxidant reduces $K_3[Fe(CN)_6]$, and subsequently Fe^{2+} generates $Fe_4[Fe(CN)_6]$, which exhibits a maximum absorption peak at 700 nm. Figure 11 illustrates the reducing ability of AOS,

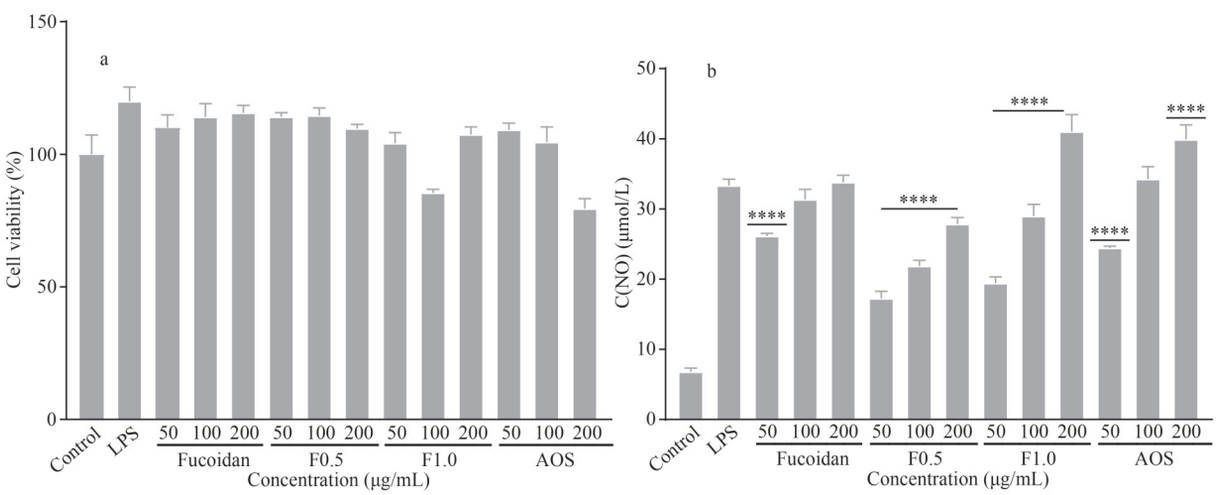

Fig.9 Effect of different components on the proliferation of RAW264.7 macrophages (a) and (b) NO production

****: P<0.000 1 vs. control.

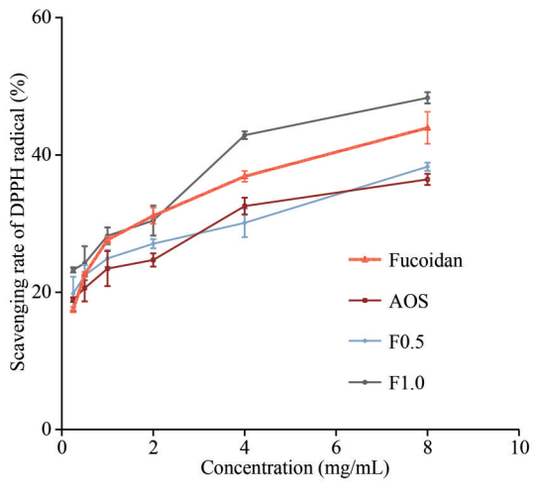

Fig.10 DPPH radical scavenging ability of different fractions

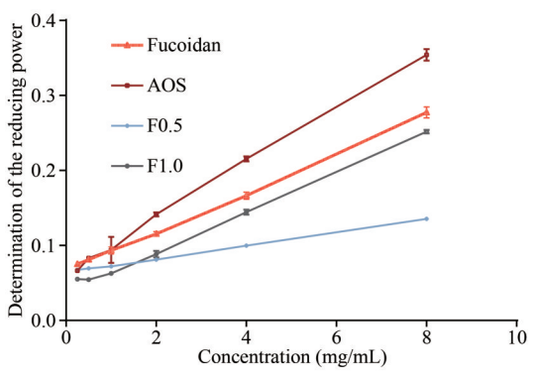

Fig.11 Reducing power of different components

fucoidan, and its fractions through the reduction with potassium ferricyanide. A higher absorbance value indicates a higher reducing ability of the sample. The reducing ability of all the samples demonstrated a concentration-dependent behavior. For instance, the OD value of fucoidan increased from 0.09 to 0.75 as the concentration increased from 0.25 mg/mL to 8 mg/mL. The reducing power of the other components was lower compared to fucoidan. In each sample, the reducing power followed the order of fucoidan>AOS>F1.0>F0.5.

4 DISCUSSION

Brown seaweeds have a complex structural arrangement and are comprised of various polysaccharides, including alginate, cellulose, laminarin, and fucoidan, as well as polyphenols, proteins, and vitamins (Leyton et al., 2016). Nevertheless, the intricate structure of seaweeds poses challenges to the degradation of their cell walls and consequently hinders the efficient isolation of these biochemical compounds. The extraction of specific compounds from algae is a laborious and expensive process. Currently, the step-by-step extraction method is the only approach for obtaining AOS and fucoidan from brown algae. The initial step involves separate extraction of fucoidan and alginate, followed by the degradation of alginate to AOS. Various traditional extraction techniques are commonly employed to extract fucoidan and alginate individually from brown algae (Daub et al., 2020). Table 3 summarizes the advantages and disadvantages of three commonly used methods for extracting fucoidan from brown algae. While traditional aqueous extraction is economical, convenient, simple to perform, and better preserves molecular properties, it is time-consuming. However, the alkaline extraction method has high extraction efficiency, it requires more stringent process conditions, which may lead to the breakage of glycosidic bonds in polysaccharides. In recent years, the enzymatic method is more commonly used. Although the polysaccharide may undergo degradation during the reaction process, the reaction conditions are mild and the extraction efficiency is high. When extracting alginate from brown algae, strong alkaline salts are generally used to convert insoluble alginate into soluble alginate. Another method involves the combined extraction of fucoidan and alginate from brown algae, followed by microbial degradation to degrade the alginate. Currently, several methods are available for the comprehensive extraction of alginate and fucoidan, such as the cellulose complex enzyme-acid precipitation method (Li et al., 2017), biorefinery method (Abraham et al., 2019), and microwave-assisted-biorefinery method (Yuan and Macquarrie, 2015). However, the biorefinery method and microwave-assisted-biorefinery method, despite their high extraction efficiency, are overly complex.

To address the limitations of the complex extraction process for AOS and fucoidan and the underutilization of brown algae, we have developed a novel extraction method by combining the alkaline digestion method with the complex enzyme hydrolysis method. In our investigation of the impact of the solid-liquid ratio on the dissolution of soluble matter, we found that the maximum dissolution of water-soluble matter in *L. trabeculata* occurred at a solid-liquid ratio of 1∶20. As the solid-liquid ratio increased, the solubility of water-soluble substances decreased, likely due to the dilution of enzyme concentration, leading to an increase in

Table 3 Comparison of three methods for extraction of fucoidan

Extraction method	Principle	Advantage	Disadvantage
Traditional water extraction	Seaweed polysaccharides are soluble in water and exhibit relatively good stability. When exposed to high temperatures, the cell walls of seaweed undergo plastic separation, allowing water to penetrate into the cells, dissolve the polysaccharides, and diffuse to the external environment (Yang et al., 2021)	This method is cost-effective, convenient, and user-friendly while also effectively preserving the molecular properties	Time consuming (Yang et al., 2021)
Alkali extraction	In alkaline solutions, there is an interaction between OH^- and cellulose in the cell wall. This interaction results in the formation of water-soluble alkali metal salts and leads to the breakage of cellulose chains. Additionally, the pH of the solution plays a role in the hydrolysis reaction of hemicellulose and proteins, further contributing to the degradation of the cell wall's integrity	The experimental conditions are mild and the extraction efficiency is high	The process requirements are stringent and exceeding the pH range required by the process may cause breakage of glycosidic bonds in polysaccharides (Dong et al., 2021)
Enzymatic extraction	Enzymes can specifically catalyze and target cell walls and cell membranes, reducing resistance from cell walls, cell membranes, and intercellular matrix during solvent extraction, allowing the release of active ingredients (Zhang et al., 2020; Wang and Chen, 2022). It can also degrade polysaccharides to low molecular weight polysaccharides (Nadar et al., 2018)	The reaction conditions are mild, the products are not easy to deteriorate, the extraction efficiency is high, the cost is low, and the environmental protection and energy saving are the advantages (Du and Wang, 2018; Xiao et al., 2019)	Enzyme extraction is specific and tends to degrade the polysaccharides during the extraction process (Nadar et al., 2018)

algal sludge production. The key factors influencing the dissolution of water-soluble substances in alkaline digestion were the concentration of Na_2CO_3, digestion time and digestion temperature. After 2 h of digestion, the cell walls are effectively broken down. However, due to the high concentration of Na_2CO_3, some initially soluble substances may slowly precipitated under high salt conditions, leading to an increase in algal sludge production. Based on the results of a one-way experiment, the optimal extraction conditions are shown in Table 1. It is worth nothing that there is limited research on fucoidan from *L. trabeculata*. Qu et al. (2014) extracted fucoidan using alcohol extraction method, resulting in a yield of 4.15%. In this study, the yield of fucoidan extracted using the method established was 14.40%, indicating that the enzyme assisted one-pot method significantly improved the yield of fucoidan. The chemical composition of fucoidan obtained using this method different from other method. In Qu's study, the fucoidan extracted from *L. trabeculata* had total sugar, fucose, and sulfate group contents of 66.38%, 21.53%, and 16.39%, respectively. In the present study, the total sugar contents of fucoidan extracted from *L. trabeculata* was much higher (83.39%), while the fucose and sulfate content were lower (15.30% and 12.53%, respectively). The monosaccharide compositions of fucoidan also differed from the study by Qu et al. (2014). In our study, the dominant monosaccharides in fucoidan were mannuronic acid and fucose, followed by xylose, which may be related to the composition of the cell walls. Xylan is the primary hemicellulose component of plant secondary walls. According to the different side chains, xylan can be divided into Glucuronoxylan (GX), 4-O-methylgucuronoxylan (MGX), Arabinoxylan (AX), and Glucuronoarabinoxylan (GAX). Togashi et al. (2009)'s study confirmed that xylan can be enzymatically hydrolyzed to produce xylose and uronic acid. Our complex enzyme hydrolysis method not only degraded the alginate into AOS, but also facilitated the breakdown of hemicellulose into xylan. The monosaccharide composition suggests that the xylan in *L. trabeculata* is compose of xylose and uronic acid. The FT-IR spectrum results demonstrated the complete decomposition of cell wall components, as evidenced by the absence of absorption peaks at 2 350/cm (Zhang et al., 2018). The FT-IR spectrum of fucoidan extracted using our method is consistent with the study of Xiao et al. (2019), indicating that the novel method did not alter the fundamental polysaccharides structure. This further confirms that the enzyme

assisted one-pot methods is a suitable approach for extracting fucoidan with high yield and intact structure.

Alginate extracted from brown algae is commonly used to prepare AOS (Aarstad et al., 2012). Various physicochemical techniques have been described for deriving AOS from alginate. Hydrolysis of alginate using endolytic alginate lyases is considered an effective strategy as it produces AOS with well-defined structures and superior functionality (Liu et al., 2019). However, there are no reports on directly preparing AOS from brown seaweeds. Therefore, in this study, a new method was developed to extract AOS directly from algae. Due to the tough cell walls of *L. trabeculata*, there is limited research on AOS, apart from a few studies related to alginate extraction. According to Fig.7, the AOS extracted using this method exhibited a double bond at the end, which is consistent with the findings of Liu et al. (2019). These enzymes hydrolyze alginate by β-elimination of glycosidic bonds, resulting in the production of unsaturated oligosaccharides with double bonds at the non-reducing end (Zhu et al., 2015). The G/M ratio of AOS extracted using the alkaline ablation-complex enzyme digestion coupling method was 1.97. Based on ESI-MS analysis, the degree of polymerization (DP) of the AOS was determined to be 3–5. From the above analysis, it can be concluded that this novel enzyme-assisted one-pot method allows for the direct preparation of AOS from algae with high yield and low, uniform DP.

Macrophages are crucial immune cells involved in modulating the immune response and chronic inflammation. RAW264.7 cells, a macrophage cell line, have been widely studied for their role in the immune system. RAW264.7 cells with M1 phenotype are known to increase nitric oxide (NO) production upon stimulation with lipopolysaccharide (LPS) and contribute to defense against pathogens and cancer cells by secreting NO and various cytokines (Franz et al., 2011). In this study, the immunostimulatory activity of fucoidan and AOS was evaluated using the ability of RAW264.7 macrophages to release NO. The experimental results showed that fucoidan, F0.5, F1.0, and AOS exhibited no cytotoxicity towards RAW264.7 cells, which is consistent with previous literature reports (Hwang et al., 2011). The immunostimulatory activity of the fucoidan, F0.5, and AOS groups was significantly higher compared to the positive control group. Among the fucoidan treated groups, the production of NO increased in the order of F1.0>F0.5, corresponding to the increase in sulfate and fucose content. Previous studies on fucoidan isolated from *Sargassum* and *Undaria* have reported a direct and positive correlation between the sulfate content of polysaccharides and their ability to induce NO release from macrophages (Cho et al., 2010).

The accumulation of reactive oxygen species leading to oxidative stress can trigger cell apoptosis and contribute to the development of chronic diseases and neurodegenerative disorders (Furman et al., 2019). In this study, the DPPH radical scavenging activity and reducing power were investigated as indicators of antioxidant capacity. Antioxidants scavenge DPPH radicals by donating hydrogen atoms, resulting in the formation of a stable DPPH molecule (Wang et al., 2010). Within the concentration range of 0–8 mg/mL, the scavenging ability of DPPH radicals increased in the order of fucoidan, F0.5, and F1.0. Based on the experimental results, it can be concluded that both the fucose and sulfate content influence the scavenging effect, with higher content leading to better scavenging activity. The relationship between the fucose and sulfate content and the scavenging ability of DPPH radicals has been previously reported in the study by Wang et al. (2010). We also evaluated the reducing powers of the samples we tested. As the concentration increased from 0.25 mg/mL to 8 mg/mL, the AOS exhibited the highest reducing power, indicating a correlation between reducing power and molecular weight size. Zhang et al. (2019) reported that AOS with a degree of polymerization (DP) of 3 showed the highest reducing properties. The effect of reducing power is due to their hydrogen-donating capacity. In this study, it is proven that AOS have strong proton donating capacity and can act as free radical inhibitors or scavengers and may act as major antioxidants. Overall, the molecular weight, fucose content, and sulfate group content showed certain effects on the antioxidant capacity. Based on our experimental data, the reducing capacity of different components may contribute to the observed antioxidant process.

5 CONCLUSION

In this study, we developed a novel enzyme-assisted one-pot method for the simultaneous extraction of AOS and fucoidan from *L. trabeculata*.

The yields and physicochemical properties of both AOS and fucoidan obtained using this method were superior to those achieved with other extraction methods. Bioactivity studies revealed that AOS, fucoidan, and their fractions exhibited immunostimulatory and antioxidant activities in vitro, suggesting their potential applications in medicine and functional food products. The bioactivity was influenced by factors such as molecular weight, fucose content, and sulfate group content. Compared to traditional extraction process, our method offers several advantages, including improved cost-effectiveness, environmental friendliness, simplicity, and effectiveness.

6 DATA AVAILABILITY STATEMENT

All data generated and/or analyzed during this study are available from the corresponding author on reasonable request.

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