



Bioactivities of *Nizamuddinina zanardinii* sulfated polysaccharides extracted by enzyme, ultrasound and enzyme-ultrasound methods

Mehdi Alboofetileh¹ · Masoud Rezaei¹ · Mehdi Tabarsa¹ · SangGuan You²

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Abstract Sulfated polysaccharide (fucoidan) was isolated from *Nizamuddinina zanardinii* by enzyme (alcalase), ultrasonic and enzyme-ultrasonic methods. The extracted fucoidans were assessed for their chemical compositions, molecular characteristics, anticancer and immunomodulatory activities. Enzyme-ultrasonic isolated fucoidan showed the maximum extraction yield (7.87%) while that obtained by ultrasonic had the minimum value (3.6%). fucoidans were composed of different levels of carbohydrates (52.78–58.65%), proteins (6.98–8.91%), sulfates (21.78–29.6%) and uronic acids (0.42–1.08%). The weight mean average molecular weight of fucoidans varied between 443.7 and 1020.85 kDa. The polysaccharide chains were consisted of fucose, galactose, glucose, mannose and xylose. All the recovered fucoidans showed strong growth inhibition against HeLa and Hep-G₂ cancer cells. The isolated fucoidans were non-toxic and considerably stimulated the macrophage cells to release nitric oxide. Enzyme extraction produced fucoidan with the most macrophage stimulation capacity (> 42 μmol). These results suggested that enzyme treatment preserved more sulfate groups in fucoidan structure influencing its anti-cancer and immunostimulatory activities.

Keywords *Nizamuddinina zanardinii* · Fucoidan · Enzyme extraction · Molecular weight · Sulfate group

Introduction

At present, consumers prefer the natural products with less additives to improve their health and life-style. In this regard, during the last decades seaweeds has been attracted the great attention of many researchers for isolation of natural substances such as polysaccharides and their sulfated derivatives, polyphenols, polyunsaturated fatty acids (PUFAs), protein, pigments, vitamins, tocopherols and phycocyanins with potential application in deferent industries like alimentary, pharmaceutical and cosmetic products (Kadam et al. 2015).

Fucoidan is a sulfated polysaccharide found in cell wall of brown seaweeds and animal species. This polysaccharide has various bioactivities including antioxidant, anti-tumor, antimicrobial, anti-viral, anti-inflammatory, antidiabetic, anti-obesity and anti-coagulant (Wang et al. 2008; Lee et al. 2012; Takeda et al. 2012; Hayashi et al. 2008; Wang et al. 2013; Kim and Lee 2013; Nishino et al. 1991; Kantachumpoo and Chirapart 2010). Fucoidan bioactivity depends on the species, seaweed compositional, structural and molecular properties, sulfate content and the purity of the final product (Cho et al. 2014; Borazjani et al. 2017). Furthermore, extraction methodology has a crucial effect on the structural characteristics and consequently the biological activities of the extracted molecules (Rostami et al. 2017).

Traditionally, the isolation of polysaccharides is mainly carried out by heating, boiling and refluxing of raw material in organic solvents. These methods suffer from several disadvantages namely long extraction time, high operation

✉ Masoud Rezaei
rezai_ma@modares.ac.ir

¹ Department of Seafood Processing, Faculty of Marine Sciences, Tarbiat Modares University, P.O.Box 46414-356, Noor, Iran

² Department of Marine Food Science and Technology, Gangneung-Wonju National University, Gangneung, Gangwon 25457, Republic of Korea

temperature, usage of high amount of organic solvents as well as low extraction yields (Cheng et al. 2015). Recently, different non-conventional extraction methods such as ultrasonic, microwave, enzymatic and supercritical fluid has been promoted in order to overcome these disadvantages. Among them, ultrasound (UAE) and enzymatic techniques gained more interests and attentions because they are highly efficient, environment-friendly, easily operational and also they can lead to the high extraction yield of natural products from raw materials (Wu et al. 2014).

Previously, various combined extraction methods have been carried out for high-added value compounds extraction from terrestrial materials such as ultrasonic-microwave extraction, ultrasound-assisted enzymatic extraction, supercritical CO_2 -microwave-hydrothermal extraction, pressurized liquids-ultrasound, and microwave-assisted enzymatic extraction (Sumere et al. 2018; Shang et al. 2018; Zeng et al. 2015; Fan et al. 2015; Cheng et al. 2015; Quitain et al. 2013). However, there are few reports and most of them recent with respect to combination of non-conventional extraction methods for isolating bioactive substances from seaweeds (Quitain et al. 2013; Guillard et al. 2015, 2016). Moreover, effects of enzymatic, UAE and combination of these techniques on the extraction yield and properties of *Nizimuddiniana zanardinii* fucoidan have not been previously reported.

Therefore, the aim of this study was to examine the combination strategy in the extraction of fucoidan from *N. zanardinii* using UAE, enzyme and enzyme-UAE methods and evaluate their implications on yield, chemical and molecular structure, anticancer and immunostimulatory properties.

Materials and methods

Materials

Nizamuddiniana Zanardinii samples were freshly collected from the rocky substrate of Chabahr in the Sistan and Baluchestan province of Iran (Oman Sea) in February 2016. The collected samples were washed, dried (40 °C), milled and maintained at freezer until use.

Extraction procedure

Pre-treatment of N. zanardinii

50 g of seaweed was suspended in 500 mL of 85% EtOH and stirred for 24 h at room temperature to remove pigments and small molecule compounds. Then the seaweed

residue was rinsed with acetone and finally dried under laminar hood at 22 ± 2 °C.

Enzyme-assisted extraction

The dried and pre-treated seaweed (50 g) was treated with alcalase (2.5 mL/dry material weight, pH 7, solid-to-solvent ratios 1:30 g/mL) for 24 h at 50 °C. Choosing the alcalase in this methods is based on our previous work about the fucoidan extraction by different carbohydrases and proteases enzymes which among them alcalase extracted fucoidan showed higher yield and better biological activity. After incubation time, the mixture was put in water bath (95 °C) for 15 min in order to inactivate the enzyme and then cooled with ice bath. After cooling, the suspension was centrifuged (10 min at 9000 rpm) and collected supernatant part was concentrated by rotary evaporator (60 °C). Alginic acid was removed by the addition of calcium chloride (1%) to the concentrated extracts after maintaining the mixture overnight at 4 °C and centrifugation (10 min at 9000 rpm). Fucoidan was precipitated by ethanol addition to reach the final concentration of 70%. Centrifugation (6000 rpm, 15 min) was used for recovering the fucoidan. The recovered fucoidan was washed with ethanol (three times) and acetone (twice) and finally dried under laminar hood (22 ± 2 °C). The fucoidan was weighed and stored at -20 °C until analyzed. The yields of fucoidan were calculated based on the dried seaweed treated with 85% EtOH and acetone.

Ultrasound-assisted extraction

The dried and pre-treated *N. zanardinii* samples were placed into a glass beaker containing distilled water (1:76 g/mL) and suspensions were sonicated by means of a high-power ultrasonic probe system with a titanium horn (frequency 20 kHz, max power 400 W, $\varnothing = 1.3$ cm) at 196 W and 70 °C for 59 min. This UAE condition is based on our previous work about optimization the fucoidan extraction by response surface methodology. The supernatant was separated from the solid part using centrifugation (9000 rpm, 10 min) and concentrated. The alginic acid was removed using calcium chloride (1%) and fucoidan was recovered with EtOH.

Enzyme-ultrasound assisted extraction

The dried and pre-treated seaweed (50 g) was initially treated with alcalase (2.5 mL/dry material weight, pH 7, temperature 50 °C, solid-to-solvent ratios 1:30 g/mL) for 23 h and then followed by sonication (power 196 W, temperature 70 °C, time 59 min). After treatment, the collected supernatant was concentrated and alginic acid

was removed using calcium chloride (1%). Finally, fucoidan was precipitated by EtOH addition to reach the final concentration of 70%.

Chemical composition

The determination of neutral sugar amount of the isolated polysaccharides was carried out using the phenol–sulfuric acid method and D-glucose as a standard, the absorbance was recorded at 490 nm (Dubois et al. 1956). Lowry method was employed to measure the amount of protein using a DC protein assay kit (Bio-Rad, CA, USA), the absorbance was recorded at 720 nm (Lowry et al. 1951). The sulfate content was determined by the BaCl₂ gelatin method and the absorbance was recorded at 360 nm (Dodgson and Price 1962). The level of uronic acid was measured by a sulfamate/m-hydroxydiphenyl assay and glucuronic acid as a standard, the absorbance was recorded at 525 nm (Filisetti-Cozzi and Carpita 1991).

Determination of monosaccharide composition

The monosaccharide composition of extracted fucoidan were determined by GC-MAS (gas chromatography mass spectrometry). Sample preparation was performed according to the procedure previously reported by Rostami et al. (2018). The monosaccharide standards including fucose, rhamnose, xylose, mannose, galactose and glucose were applied according to the reference.

FT-IR spectroscopy

For FT-IR analysis, sulfated polysaccharides were mixed with KBr powder and pressed into a pellet. Then, the pellets were scanned using a FT-IR spectrophotometer (Bruker Instruments, Billerica, USA) in the wavelength of 400–4000 cm⁻¹ at room temperature.

Determination of molecular properties

Molecular properties of the sulfated polysaccharides were determined by HPSEC–UV–MALLS–RI system. Sample preparation was performed according to procedure previously reported by Bahramzadeh et al. (2019). Molecular properties including average molecular weight (M_w), number average molecular weight (M_n), polydispersity and radius of gyration (R_g) of extracted fucoidan were determined using ASTRA 5.3 software (Wyatt Technology Corp.). Following equation was used for calculation of the specific volume of gyration (SV_g):

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

in which N is Avogadro's number ($6.02 \times 10^{23}/\text{mol}$) and the units for SV_g , M_w and R_g were cm³/g, kDa and kDa respectively.

Scanning electron microscopy (SEM)

SEM analysis (Philips XL 30, Netherlands) at a 20.0 kV accelerating voltage was applied to investigate the effect of different extraction methods on the microstructure of extracted fucoidan. Samples first coated with gold and then observed under different magnifications.

Anticancer activity

HeLa and HepG₂ cells were plated (1×10^4 cells/well) in a 96-well plate and the plates were put in incubator (4 h, 37 °C, 5% CO₂). After incubation, different concentrations of polysaccharide samples (100, 200 and 400 µg/mL) were added to each well and then plates put again in the incubator (72 h, 37 °C, 5% CO₂). Finally, the WST-1 colorimetric assay kit was employed for determination the anticancer activity of the samples. 5-fluorouracil (5-Fu, 10 µg/mL) was considered as a positive control.

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% CO₂. After incubation, 100 µL of the polysaccharide samples (10, 25 and 50 µg/mL) were added to each well and plate incubated at 37 °C. After 3 days 20 µL of WST-1 solution were added to each well and plates incubated at 37 °C. After 4 h absorbance of samples was determined at 450 nm using a microplate reader (Borazjani et al. 2018).

RAW264.7 cells were seeded (1×10^5 cells/well) in a 96-well plate and treated with different concentration of polysaccharide (10, 25 and 50 µg/mL) and lipopolysaccharide (LPS, 1 µg/mL). After incubation at 37 °C for 18 h, the separated supernatant was mixed with Griess reaction solution and maintained in room temperature. After 10 min, absorbance of samples was determined at 540 nm using a microplate reader. Nitric oxide (NO) production of macrophage cells was quantified by matching with a sodium nitrite standard curve (Green et al. 1982).

Statistical analyses

All experiments were expressed as mean values \pm standard deviations. One way ANOVA and Duncan's test ($p < 0.05$) was performed for the calculating the

differences between the extraction methods and concentrations of Fucoidan.

Results and discussion

Effect of extraction methods on the yield of fucoidan

The effect of different extraction methods (enzyme, UAE, enzyme-UAE) on fucoidan yields is shown in Table 1. The type of extraction clearly affected the fucoidan yield and it varied from 3.6 to 7.87%. Among the tested methods, enzyme-UAE exhibited the highest fucoidan yield (7.87%) while the lowest value was obtained in the UAE extraction (3.6%). The higher yields in EAE (5.58%) compared to UAE could be due to better disintegration of cell wall matrix by alcalase enzyme in a rather prolonged reaction time. You et al. (2013) reported that enzyme-assisted method (EAE) had the higher *Cornus officinalis* polysaccharides yield compared to UAE methods. Combination the enzyme and UAE led to achieve the heights fucoidan yields (7.87%). The higher yields of the enzyme-UAE compared to those of the EAE and UAE could be explained by the fact that when alcalase was added into the extraction suspension, initially the enzyme catalyzes the cell wall and then sonication produces high cavitation intensity leading to more solvent penetration. These sequential events can accelerate the release of intracellular polysaccharide into the solvent and consequently increase the extraction efficiency. Synergistic effect between enzymes and UAE for extraction polysaccharides was reported by Easson et al. (2011) and Wu et al. (2014). Overall, results of the current study showed that the enzyme-UAE could be an appropriate and effective extraction technique for the isolation of fucoidan from *N. zanardinii* with high extraction yield.

Table 1 Yield, chemical and monosaccharide composition of different fucoidans isolated from *N. zanardinii*

Fucoidan	Yield (%)	Protein (%)	Carbohydrate (%)	Uronic acid (%)	Sulfate (%)
UAE	3.6 ± 0.31 ^c	8.53 ± 0.45 ^a	58.65 ± 0.17 ^a	1.08 ± 0.06 ^a	22.97 ± 0.29 ^b
Enzyme	5.58 ± 0.35 ^b	6.98 ± 0.25 ^b	53.55 ± 0.17 ^b	0.42 ± 0.02 ^c	29.6 ± 0.6 ^a
Enzyme-UAE	7.87 ± 0.72 ^a	8.91 ± 0.25 ^a	52.78 ± 0.27 ^b	0.73 ± 0.02 ^b	21.78 ± 1.11 ^b
Fucoidan	Monosaccharide composition				
	Fucose (%)	Galactose (%)	Glucose (%)	Mannose (%)	Xylose (%)
UAE	32.33 ± 0.16 ^b	29.95 ± 0.25 ^a	3.19 ± 0.06 ^a	27.97 ± 0.11 ^a	6.56 ± 0.13 ^a
Enzyme	32.93 ± 0.37 ^{ab}	30.8 ± 0.20 ^a	2.54 ± 0.24 ^b	26.75 ± 0.83 ^a	6.97 ± 0.40 ^a
Enzyme-UAE	33.86 ± 0.57 ^a	31.32 ± 0.81 ^a	2.04 ± 0.24 ^b	27.31 ± 0.21 ^a	5.47 ± 0.18 ^b

The letters a,b,c,d indicate significant difference at $p < 0.05$

Chemical compositional analysis

The contents of carbohydrate, protein, uronic acid and sulfates in *N. zanardinii* fucoidans extracted by enzyme, UAE and enzyme-UAE methods are showed in Table 1. The carbohydrate contents in fucoidan extracted by enzyme, UAE and enzyme-UAE were 53.55%, 58.65% and 52.78%, respectively. As can be seen in Table 1, UAE (8.53%) and enzyme-UAE (8.91%) fucoidans contained higher amounts of proteins compared with that of EAE (6.98%). Uronic acid contents in enzyme, UAE and enzyme-UAE were 0.42%, 1.08% and 0.73%, respectively. The sulfate content was the highest in enzyme (29.6%), followed by UAE (22.97%) and enzyme-UAE (21.78%). This discrepancy in the chemical composition of different fucoidans might be related to the type of extraction method that used (Dong et al. 2016).

Monosaccharide compositions of enzyme, UAE and enzyme-UAE extracted fucoidans are shown in Table 1. In all extracted fucoidans the fucose was the main monosaccharide and other monosaccharides are in the following order from high to low content: Mannose > Galactose > Xylose > Glucose. Rhamnose and Arabinose were not found in the extracted fucoidans. This monosaccharide composition was reported previously for fucoidan extracted from *Sargassum augustifolium* with different ratios (Borzajani et al. 2018). These results indicated that not only extraction yields differ from one isolation technique to another, but also their chemical constituents are significantly divergent.

Infrared spectroscopy analysis

Figure 1a show the infrared spectra of fucoidans extracted by enzyme, UAE and enzyme-UAE from *N. zanardinii*. Like the other seaweed fucoidans, the FT-IR spectra of different fucoidans showed a strong absorbance band at 3424 cm^{-1} that is attributed to O–H stretching vibration.

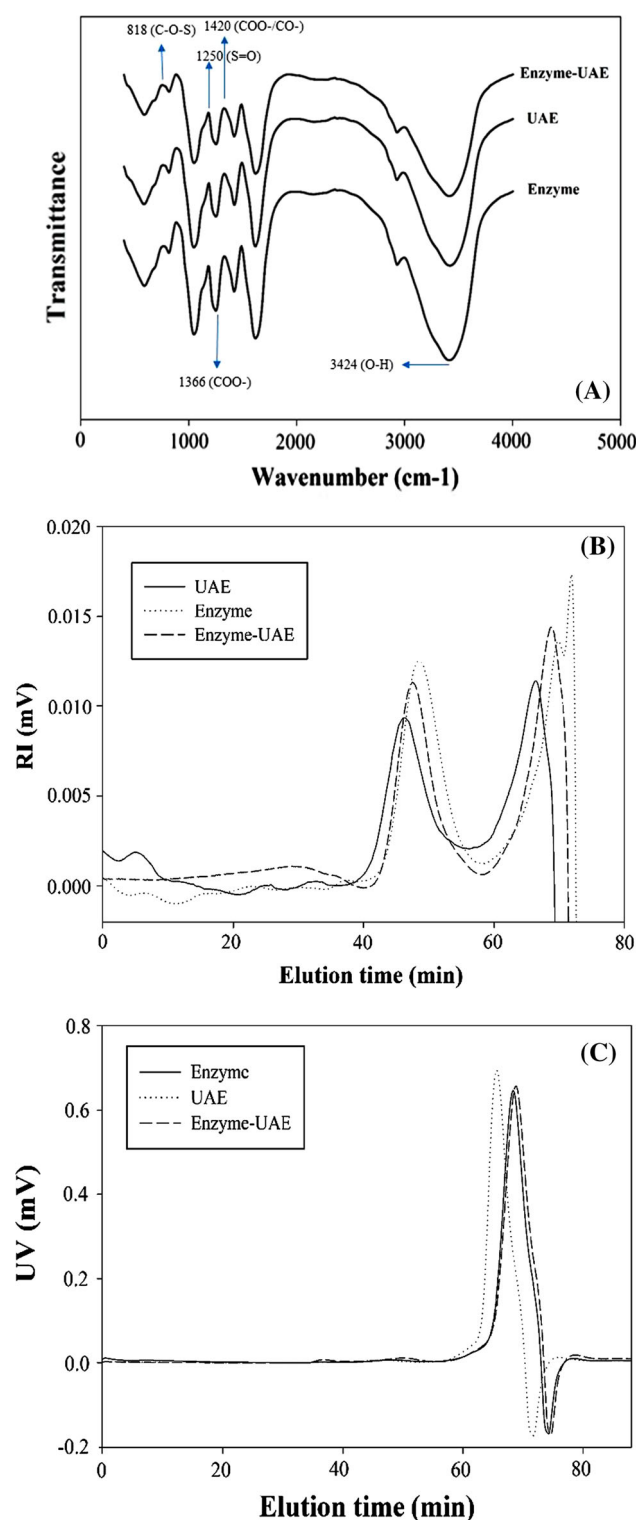


Fig. 1 FT-IR spectra (a), RI (b) and UV chromatograms (c) of different fucoidans

The band at 1420 cm⁻¹ and 1366 cm⁻¹ represent the presence of the asymmetrical bending vibration of CH₃, and symmetrical bending vibration of CH₃, respectively (Lim et al. 2014). The band at 1250 cm⁻¹ and 818 cm⁻¹

were attributed to the sulfate esters (S=O) and sulfate group (C-O-S), respectively (Huang et al. 2016).

Molecular characteristics

The RI superimposed chromatograms for fucoidans extracted by enzyme, UAE and enzyme-UAE methods are showed in Fig. 1b. As shown in the RI chromatogram, the extracted fucoidans had one major peak at the elution times of 42–55 min. The peak at the elution time of 66 min was related to the calcium chloride. As can be seen in Fig. 1c, enzyme extracted fucoidans had lower UV peak levels than those extracted by UAE and enzyme-UAE, corresponding to their protein contents. This result indicated that the enzyme treatment removed the proteins from the extracted fucoidan more than to the others. These results were in agreement with protein content of extracted fucoidan. The average molecular weights of enzyme, UAE and enzyme-UAE were 642.85, 1020.85 and 443.70 kDa, respectively (Table 2). The R_g values, which indicate the size of the molecules, of enzyme, UAE and enzyme-UAE were 56.5, 62.05 and 52.05 nm, respectively. The SV_g values for different fucoidan ranged from 0.29 to 0.80 cm³/g. It could be seen that UAE had the relatively more compact compared to enzyme and enzyme-UAE.

Morphological analysis

Figure 2 presents the scanning electron micrographs (SEM) of fucoidans extracted by enzyme, UAE and enzyme-UAE at different magnifications (200, 500 and 1000 fold exaggeration conditions). As can be seen, under a 200 fold exaggeration condition, all fucoidans exhibited a distributed fluffy powder. They also showed an irregular shape with no uniform size and plenty of pores at 500 and 1000 fold exaggeration.

Anticancer activity

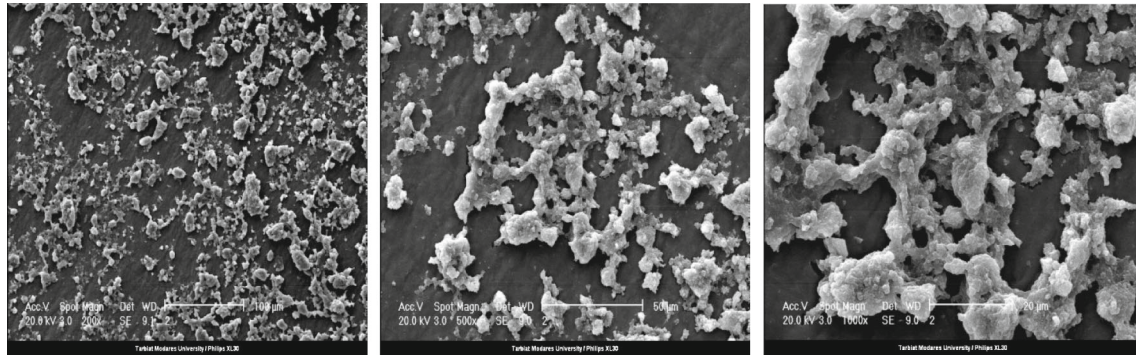
In the present study, the anticancer activities of fucoidans extracted from *N. zanardinii* by enzyme, UAE and enzyme-UAE were tested at three concentrations ranging from 100 to 400 µg/mL. The fucoidans showed the anticancer activity ranging from 28.40 to 68.46% for HeLa cells and 24.72 to 57.29% (Fig. 3a) for Hep-G₂ cells (Fig. 3b). The anticancer activity of the fucoidan extracted by UAE was lower than those extracted by enzyme and enzyme-UAE. A previous report (Zhao et al. 2016) showed that anticancer activity of bioactive polysaccharides is attributable to the various factors such as sulfate content, monosaccharide composition, molecular weight and amount of impurity of protein and total phenol. Also, Yang et al. (2008) proposed that anticancer activities of brown

Table 2 Weight average molecular weight (M_w), number average molecular weight (M_n), radius of gyration (R_g), specific volume of gyration (SV_g) and polydispersity of different extracted Fucoidan

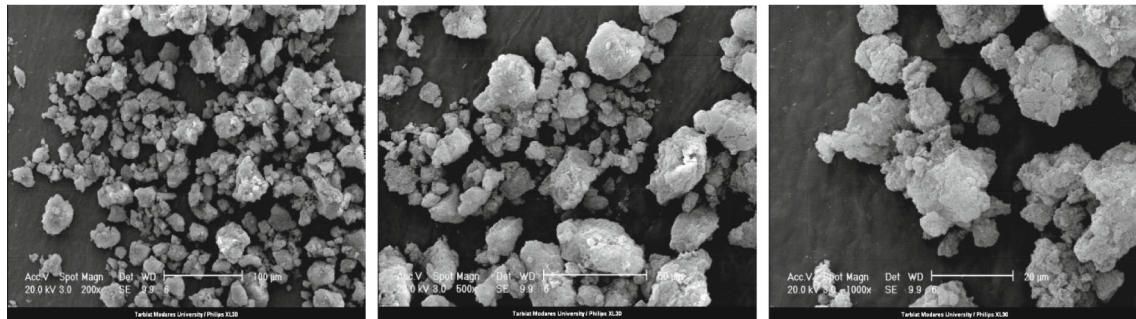
Fucoidan	M_w (kDa)	M_n (kDa)	Polydispersity (M_w/M_n)	R_g (nm)	SV_g (cm ³ /g)
UAE	1020.85 ± 31.75 ^a	806.15 ± 17.47 ^a	1.27 ± 0.01 ^a	62.05 ± 4.03 ^a	0.29 ± 0.01 ^c
Enzyme	642.85 ± 7.85 ^b	642.2 ± 13.44 ^b	1 ± 0.01 ^b	31.05 ± 0.91 ^c	0.41 ± 0.03 ^b
Enzyme-UAE	443.7 ± 52.61 ^c	345.7 ± 34.93 ^c	1.28 ± 0.02 ^a	52.05 ± 0.83 ^b	0.80 ± 0.05 ^a

The letters a,b,c,d indicate significant difference at $p < 0.05$

Enzyme



UAE



Enzyme-UAE



Fig. 2 Scanning electron micrographs of the different fucoidans (× 200; × 500; × 1000, left to right respectively)

seaweed fucoidans depend on species and growing conditions of brown seaweeds, extraction and purification

methods and as well as the use of different cancer cell lines.

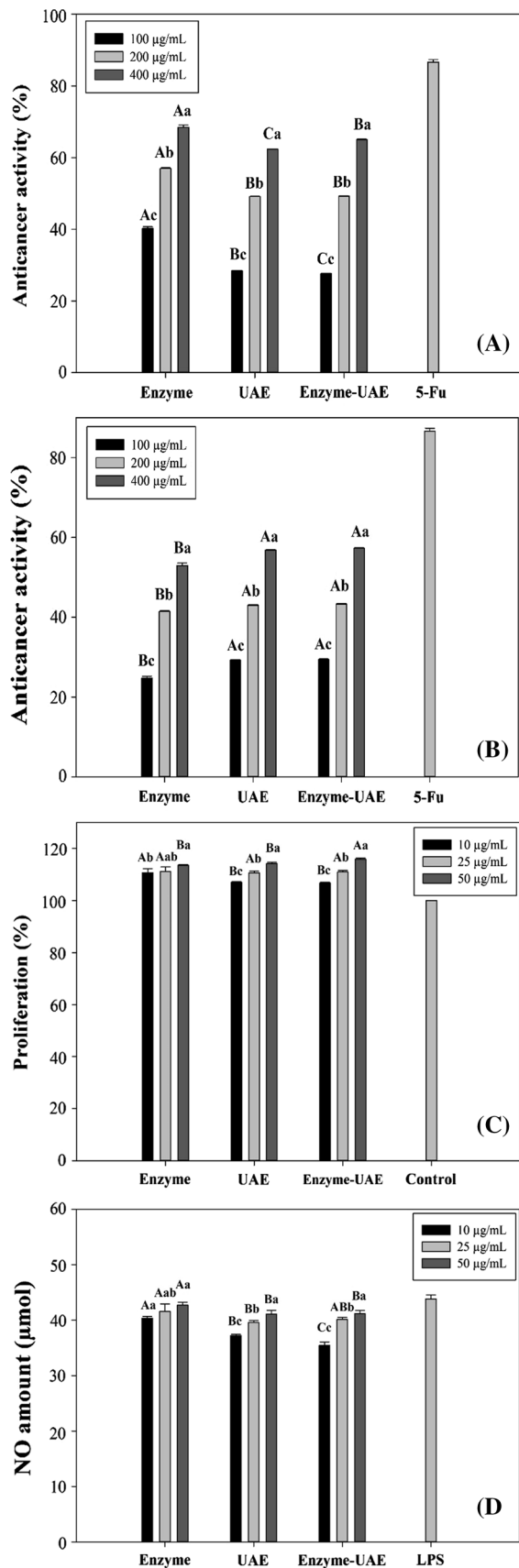


Fig. 3 Effects of different fucoidans on proliferation of HeLa (a) and HepG₂ cancer cells (b), proliferation (c) and NO production (d) in RAW264.7 macrophages cells (mean ± SD). The letters a, b, c, d indicate a significant difference ($p < 0.05$) between the concentrations of each fucoidan, and A,B,C,D indicate a significant difference ($p < 0.05$) between different fucoidans at a constant concentration

Immunomodulatory activity

As can be seen in Fig. 3c, compared to the control proliferations of RAW264.7 cells were significantly increased in the presence of all the extracted fucoidans. These results suggested that *N. zanardinii* sulfated polysaccharides are nontoxic and could stimulate the growth of RAW 264.7 cells. The nitric oxide (NO) productions of RAW264.7 cells in the presence of different concentrations of different fucoidans (10, 25 and 50 µg/mL) were investigated and the results are shown in the Fig. 3d. When RAW264.7 cells were cultured in medium containing enzyme-UAE, a minimum amount of NO was released, whereas treating the cells with enzyme extracted fucoidans resulted in significant increase of NO production. The higher stimulating potential of fucoidan isolated using enzyme could be attributed to its greater sulfate content. The direct and favorable relationships between sulfate contents of polysaccharides with their capacities to induce macrophage NO release have been previously reported in fucoidans isolated from *Sargassum angustifolium* and *Undaria pinnatifida* (Borazjani et al. 2018; Cho et al. 2011).

Conclusion

The yields, chemical composition, molecular properties, anticancer and immunomodulatory activities of fucoidan extracted from *N. zanardinii* by enzyme, ultrasound and enzyme-ultrasound methods were evaluated. The highest and lowest yields were obtained by enzyme-ultrasound and ultrasound methods, respectively. Different extraction methods resulted in obtaining fucoidans with various chemical compositions and molecular weights. Enzyme-ultrasound isolated fucoidans showed the lowest amount of sulfate group while those obtained by enzyme had the highest levels of sulfate content. Fucose, galactose, mannose, glucose and xylose were the monosaccharides present in all extracted fucoidans. All the isolated fucoidans exhibited appropriate macrophage stimulating properties and also anticancer activity against HeLa and HepG₂ cells in vitro.

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