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Research Article

Sequential ultrasonic/microwave assisted extraction of fucoidan from *Nizamudinia zanardinii* and evaluation of its biological activities

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Abstract

In the current study, the combination of ultrasound and microwave methods (UM) has been used for the extraction of fucoidan from *Nizamuddinia zanardinii* and the effect of this method on the chemical and monosaccharide composition, molecular weight, cytotoxic (against human cervical cancer cell (HeLa) and hepatocellular carcinoma cell ($HepG_2$)), and immunomodulatory activities of the recovered fucoidan have also been investigated. The fucoidan yield under UM method was 5.53%. The extracted fucoidan chemically included 45.87% carbohydrate, 10.17% protein, 27.16% sulphate, and 1.1% uronic acid. The fucose (35.65%), mannose (28.94%), galactose (26.35%), xylose (7.74%), and glucose (1.33%) were the main monosaccharides of the extracted fucoidan. The average molecular weight of fucoidan was 748 kDa. The results also demonstrated that the extracted fucoidan had 62.41-78.08% and 62.45-70.29 % cytotoxic activity for HeLa and $HepG_2$ cells, respectively. The nitric oxide (NO) production of RAW264.7 cells was increased with increasing the concentration of fucoidan and maximum NO production was found to be 37.79 µmol at 50 µg/mL.

Keywords: Seaweeds, *Nizamuddinia zanardinii*, Fucoidan, Biological activities, Extraction methods

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Introduction

Cancer is a chronic disease and considered one of the main causes of death worldwide. GLOBOCAN 2020 estimated that the number of new cancer cases reached 19.3 million globally, and nearly 10 million people died from cancer in 2020 (Ferlay *et al.,* 2021). Nowadays, chemotherapy is widely used for the treatment of various cancers. However, it causes a wide range of negative side effects (Schirrmacher, 2019). Based on these, identify and development of potent natural anticancer agents without any side effects has received much attention.

Marine organisms are a rich source of natural products with different biological activities such as anticancer, antioxidant, antiviral, anti-inflammatory, antihypertensive, antibiotic, anticoagulant, *etc* (Wijesekara *et al.*, 2011). Among marine organisms, seaweeds contain different compounds such as polysaccharides, peptides, polyphenols, fatty acids, and vitamins with different bioactivities (Palanisamy *et al.*, 2017).

Fucoidan is a sulfated polysaccharide of brown seaweeds and it is mainly constituted of fucose sugars accompanied by lower levels of mannose, galactose, and xylose (Bilan *et al.,* 2016). Fucose is a hexose deoxy sugar with the chemical formula $C_6H_{12}O_5$ and is the fundamental subunit of the fucoidan polysaccharide (Wijesinghe and Jeon, 2012). Although, the interconnections of fucoidan units differ among various brown seaweeds, they are mostly formed of the main backbone consisting of either (1→3)-α-L-fucopyranose or alternating (1→3)- and (1→4)-α-L-fucopyranose residues (Bahramzadeh *et al*., 2019). Fucoidans offers a number of different bioactivities including antibacterial, antiviral, antioxidant, anti-inflammatory, antitumor, anti-diabetic, anticoagulant, antithrombotic and immunomodulatory activities (Usoltseva *et al.,* 2018). Anticancer activities of fucoidans were documented in previously published papers and in some cases, they exerted promising anticancer activities. Generally, fucoidans can directly kill cancer cells through apoptosis, antiangiogenesis, and inhibiting the cellular migration and enhancing various immune responses (Deniaud-Bouët *et al.,* 2017). Until now, fucoidans showed antiproliferative activities on different cancer cell lines such as A549, MCF-7, MDA-MB-231, PC-3 and HCT-15 cell lines and *etc* (Vo and Kim, 2013). On other hand, *in vivo* studies have also shown that fucoidans are able to confront cancers via enhancing immunity by activating macrophages, T cells, B cells, natural killer cells (NK) and cytotoxic T cells. Among the components of the immune system, macrophages play a key role in the response of innate immunity to cancer cells by releasing nitric oxide (NO) or cytokines (Borazjani *et al.,* 2018).

It has been documented that extraction method greatly affects the structural properties and activities of fucoidans (Sun *et al.,* 2018). Conventional extraction methods of fucoidans had some disadvantage such

as time consuming and high energy demanding methodologies with low extraction efficiency (Saravana *et al.*, 2018). As industry prefer the faster, greener and cheaper methods for the extraction of bioactive compounds, in the last decades several novel and/or advanced technologies have been developed such as supercritical fluid, subcritical water, ultrasound, microwave, pulsed electric field and enzyme digestion to minimize the disadvantages of conventional extraction methods (Grosso *et al.*, 2015). Combination of these methods may offer further advantages. However, the effect of combined methods on the structure and bioactivities of target compounds should be investigated.

Based on this, the aim of the current study was to evaluate the extraction process of fucoidan from *N. zanardinii* using combination of ultrasound and microwave methods (UM) and investigate the chemical and monosaccharide composition, molecular weight, anticancer and immunomodulatory activities of extracted fucoidan.

Materials and methods

Chemicals

Calcium chloride ($CaCl_2$), Sodium nitrate ($NaNO_3$), Potassium bromide (KBr), Sodium azide (NaN_3), Barium chloride ($BaCl_2$), phosphate buffered saline (PBS) and 5-fluorouracil (5-Fu) were purchased from Sigma–Aldrich (St.Louis, MO, USA). The HeLa and $HepG_2$ cells were purchased from the American Type Culture Collection (Manassas, Va., USA). RPMI-1640 medium was from Gibco BRL, Life Technologies (Gaithersburg, Md., USA). Penicillin, streptomycin, and fetal bovine serum (FBS) were obtained from Hyclone (Logan, Utah, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Merck Chemical Co. (Darmstadt, Germany). Acetone and ethanol were from Chem-Lab (Zedelgem, Belgium) and Taghtir Khorasan (Mashhad, Iran) companies, respectively.

Preparing and pretreatment of seaweed materials

The naturally grown *N. zanardinii* samples were freshly collected during the winter season from the coastal areas of Chabahr, a city in the Systan-o-Bluchestan province, South of Iran. After the collection, the seaweeds were cleaned and transferred to the laboratory. In the laboratory, the seaweed samples were again washed with tap water and dried in an oven (40°C) for 3 days. Dried seaweed was powdered and stored in the freezer (-20°C). For pretreatments of seaweed samples, initially, the powered seaweeds were treated with ethanol (EtOH, 85%, 1:10 g/mL ratio) with constant mechanical stirring overnight at room temperature to remove pigments and low molecular weight compounds. Then, the solid part was recovered and washed with acetone several times and dried overnight at room temperature (Yang *et al.*, 2008).

Sequential ultrasound/microwave assisted extraction of fucoidan

The dried and pretreated seaweed was initially treated with ultrasound (200 W, 50°C, solid-to-solvent ratios 1:30 g/mL, 20 min) and then followed by microwave (700 W, 90°C, 20 min). After treatment, the supernatant was collected by centrifugation (3600g, 10 min, room temperature) and concentrated by a rotary evaporator (50°C). Alginic acid was removed from the concentrated supernatants using calcium chloride (1%). Finally, fucoidan was precipitated by EtOH addition to reach the final concentration of 70%. The precipitated fucoidan was recovered by centrifugation and washed three times with EtOH and twice with acetone and dried at room temperature. The dried fucoidan was stored in a freezer until experiments (Zeng *et al.*, 2015 with some modifications).

Fourier-transform infrared spectroscopy analysis (FT-IR)

The dried fucoidan was mixed with KBr powder and then pressed into a disk for FT-IR measurement. The IR spectra of the fucoidan were determined using a Fourier transform IR spectrophotometer (Bruker Instruments, Billerica, USA) in the frequency range of 400–4000 cm^{-1} (Palanisamy *et al.*, 2017).

Chemical compositions

Carbohydrate, proteins, sulfate, and uronic acid content of the extracted fucoidan were determined by phenol–H_2SO_4 colorimetric (Dubois *et al.*, 1956), Lowry (Lowry *et al.*, 1951), $BaCl_2$ gelatin (Dodgson and Price, 1962) and m-hydroxybiphenyl methods (Filisetti-Cozzi and Carpita, 1991), respectively.

Monosaccharide composition

Monosaccharide composition of the extracted fucoidan was analyzed by GC-MS (gas chromatography–mass spectrometry) under the conditions previously reported by Tabarsa *et al.* (2015). Briefly, 60 mg of fucoidan samples were placed in a sealed glass tube and completely hydrolyzed with 4 M trifluoroacetic (TFA) acid at 120°C for 5 h. After hydrolysis, the TFA was removed with a dried stream of nitrogen at 50°C. The hydrolyzed products were reduced by sodium borohydride and acetylated with acetic anhydride and the final derivatives were analyzed by GC-MS. The monosaccharide standards, including rhamnose, xylose, mannose, galactose, arabinose, and glucose were applied according to reference.

Molecular properties

Molecular weight of the extracted fucoidan was determined by HPSEC–UV–MALLS–RI system (high-performance size exclusion chromatography column coupled to UV, multi-angle laser light scattering, and refractive index detection) under the conditions previously reported by Anvari *et al.* (2016). Briefly, 4 mg of fucoidan was dissolved in 2 mL of distilled water and heated in a microwave oven for 30s and then filtered through a cellulose acetate membrane

(3.0 m pore size; Whatman International) before injection into the HPSEC–UV–MALLS–RI system. The column used was a high performance size exclusion chromatography column (TSK G5000 PW, 7.5mm × 600 mm, Toso Biosep, Montgomeryville, PA, USA). 0.15 M $NaNO_3$ and 0.02% NaN_3 were used in the mobile phase at a flow rate of 0.4 mL/min. The normalization of the MALLS detector and the determination of volume delay among UV, MALLS, and RI detectors were carried out with bovine serum albumin. The average molecular weight (M_w) of the fucoidan was calculated with ASTRA 5.3 software.

Cytotoxic activity

The cytotoxic activity of the fucoidan was examined using human HeLa and $HepG_2$ cell lines. Cancer cells were seeded in a 96-well microplate and incubated for 4 h at 37°C in the presence of 5% CO_2. Then, different concentrations of fucoidan solutions (100, 200, and 400 µg/mL) or 5-fluorouracil (5-Fu; 10 µg/mL, as positive control) were added to each well and the cell cultures were incubated for 72 h at 37°C. After incubation, the anticancer activity of the fucoidan was determined using the WST-1 colorimetric assay kit (Roche Diagnostics, Madison, WI, USA) (Borazjani *et al*., 2018).

Immunomodulatory activity

RAW264.7 cells were seeded (1×10^4 cells/well) in 96-well plates with RPMI-1640 medium containing 10% FBS and incubated for 24 h at 37°C with 5% CO_2. After incubation, 100 µL of the fucoidan samples (10, 25, and 50 µg/mL) were added to each well and plate incubated at 37°C. Then, 20 µL of WST-1 solution was added to each well and the plates were incubated at 37°C. After 4 h absorbance of samples was determined at 450 nm using a microplate reader (Borazjani *et al*., 2018).

The immune-enhancing activity of the fucoidan was determined on the basis of nitric oxide (NO) production in RAW264.7 cell culture supernatants. For this, RAW264.7 cells were seeded (1×10^5 cells/well) in a 96-well plate and treated with different concentrations of polysaccharide (10, 25, and 50 µg/mL) and lipopolysaccharide (LPS, 1 µg/mL, as positive control). After incubation at 37°C for 18 h, the separated supernatant was mixed with Griess reaction solution and maintained at 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 data were reported as mean ± SD. SPSS statistic program (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL, USA) was used for data analysis. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to determine significant differences between variables.

Results

FT-IR spectroscopy

The FT-IR pattern of the extracted fucoidan is shown in Figure 1. As can be seen, different absorption peaks were recorded in the range of 400–4,000 cm^{-1} for fucoidan extracted using UM method. FT-IR pattern included a strong absorbance peak at 3424 cm^{-1} from the O-H stretching vibration, a peak at 1620 cm^{-1} from the asymmetrical bending vibration of CH_3, a peak at 1420 cm^{-1} from the symmetrical bending vibration of CH_3, a peak at 1250 cm^{-1} from the sulfate esters (S=O), and a peak at 818 cm^{-1} from the sulfate group (C-O-S).

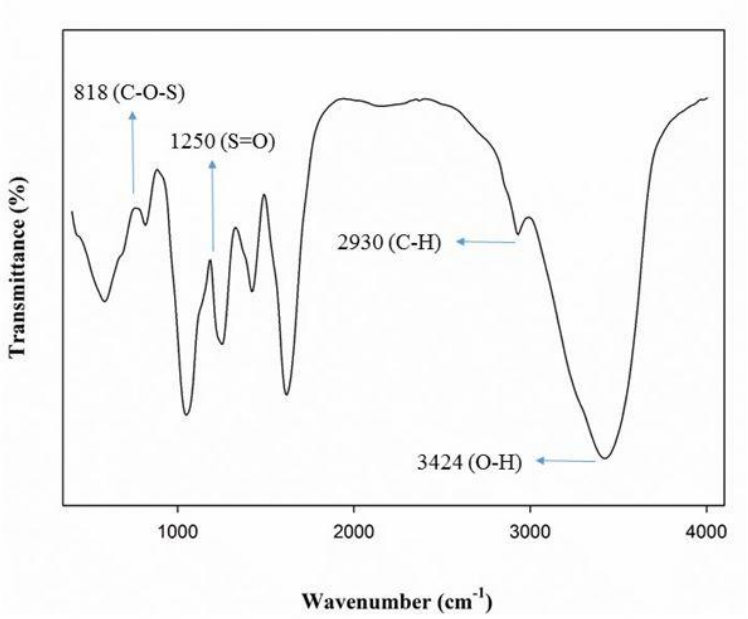

Figure 1: FT-IR spectra of fucoidan extracted by ultrasonic/microwave (UM) method.

Fucoidan yield and characterization

The extraction yield of fucoidan from *N .zanardinii* samples using UM method was 5.53%. The extracted fucoidan chemically contain 45.87% carbohydrate, 10.17% protein, 27.16% sulphate and 1.1% uronic acid.

Monosaccharide composition of the extracted fucoidan were fucose (35.65%), mannose (28.94%), galactose (26.35%), xylose (7.74%) and glucose (1.33%). The results also showed that rhamnose and arabinose were not found in the fucoidan extracts. The molecular weight of extracted fucoidans was 748 kDa.

Cytotoxic activity

Figure 2 shows the cytotoxic activity of the UM-extracted fucoidan against HeLa (A) and $HepG_2$ (B) cells. At the tested concentrations (100-400 µg/mL), the cytotoxic activity of fucoidan varied from 62.41 to 78.08% for HeLa cells and from 62.45 to 70.29% for $HepG_2$ cells. The highest inhibition by means of cell viability percentage (21.92% for HeLa and 29.71% for $HepG_2$ cells) was recorded at 400 µg/mL of fucoidan.

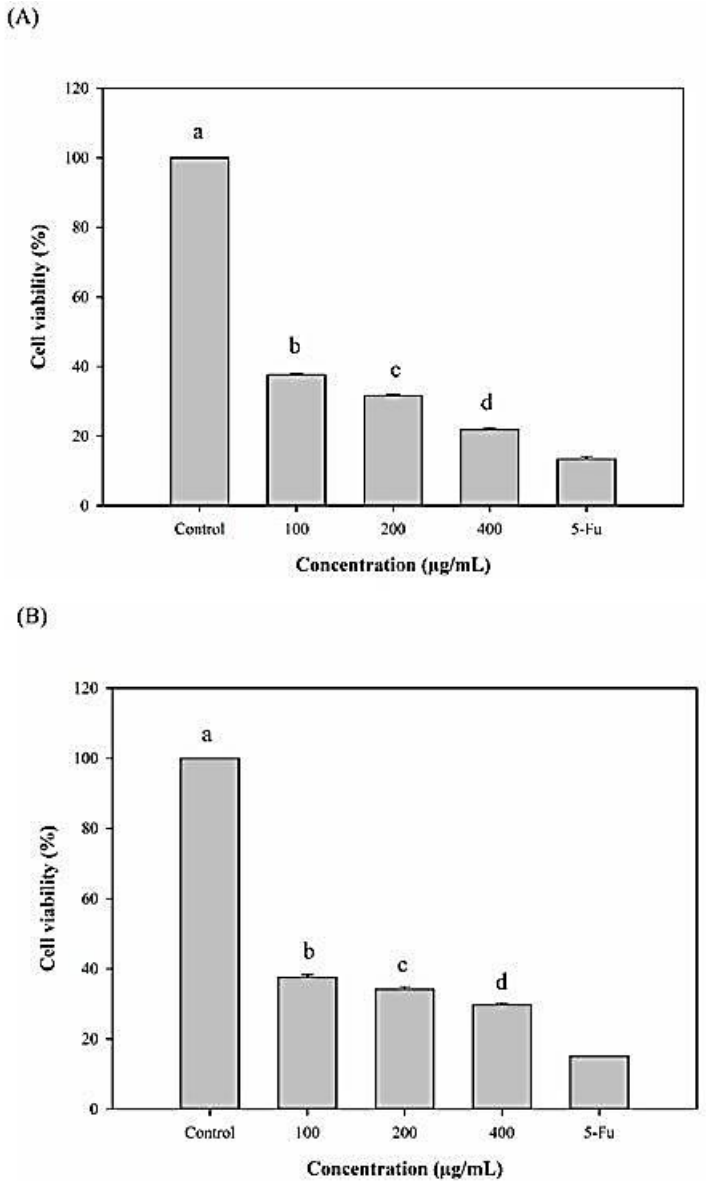

Figure 2: Effects of UM-extracted fucoidan on the proliferation of HeLa (A) and $HepG_2$ (B) cancer cells compared with the controls (n=3, means±SD). The letters a, b, c indicate a significant difference (p<0.05) between the concentrations of the fucoidan.

Immunomodulatory activity

Immunomodulatory activity of the UM-extracted fucoidan from *N. zanardinii* on RAW 264.7 cell was presented in Figure 3. As can be seen in Figure 3A, the extracted fucoidan not only was not cytotoxic to RAW264.7 cells but also promoted the proliferation of cells compared to the control group. The effect of extracted fucoidan on the nitric oxide (NO) production of murine macrophage RAW264.7 cells at the concentration ranging from 10–50 µg/mL was presented in Figure 3B. NO production from RAW264.7 cells in the presences of UM-extracted fucoidan was 35.97, 36.92 and 37.79 µmol at 10, 25 and 50 µg/mL, respectively.

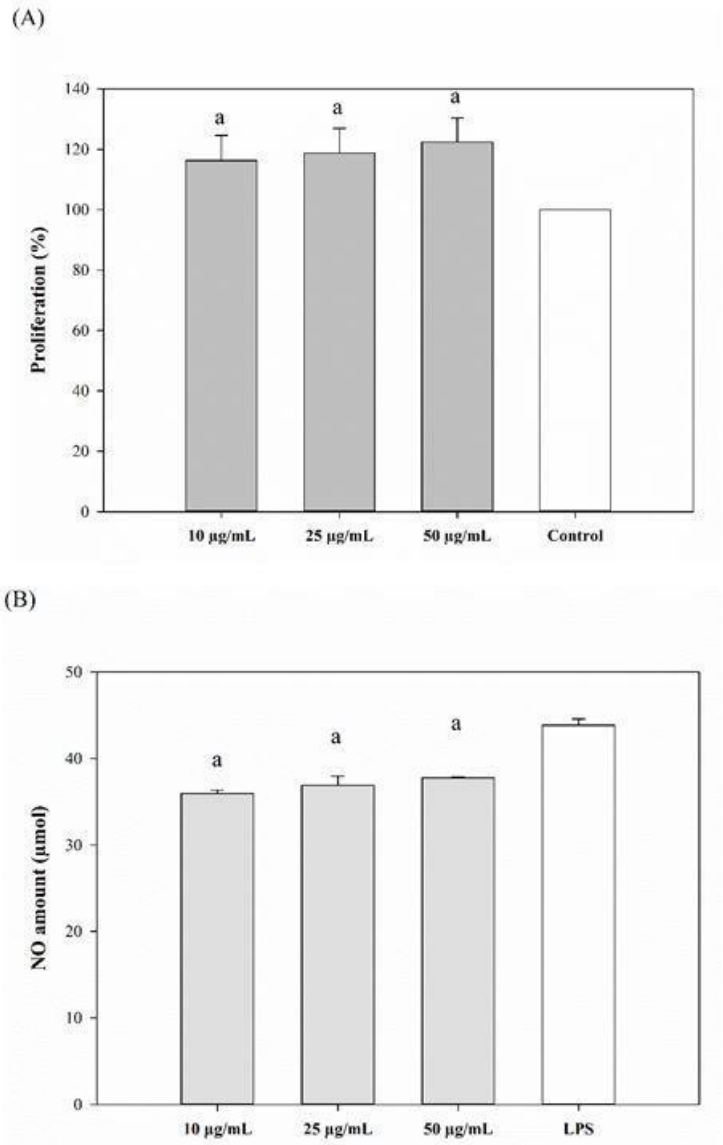

Figure 3: Effects of UM-extracted fucoidan on proliferation (A) and NO production (B) in RAW264.7 macrophage cells (n=3, means±SD). The letters a, b, c indicate a significant difference (p<0.05) between the concentrations of the fucoidan.

Discussion

The fucoidan yield from *N. zanardinii* by UM method (5.53%) was higher than the fucoidan content of species such as *Alaria marginata* (1.4%, Usov *et al.*, 2001), *Scytosiphon lomentaria* (2.8%, Usov *et al.*, 2001) and *Padina sp.* (2.06%, Lim *et al.*, 2014) but lower than those obtained from *S. glaucescens* (9.83%, Huang *et al.*, 2016), *Nemacystus decipients* (16.67%, Li *et al.*, 2017) and *Undaria pinnatifida* (25.4-69.9%, Mak *et al.*, 2013). These discrepancies in the yields of fucoidans from brown seaweeds are believed to have originated from the differences in the species, age, seasons, and locations of the seaweed sampling (Usov *et al.*, 2001; Zvyagintseva *et al.*, 2003; Rioux *et al.*, 2009; Lim *et al.*, 2014). The type of isolation and purification method used to extract the polysaccharide could also

have a determinant effect on the polysaccharide yield (Ale *et al.,* 2012; Azmir *et al.,* 2013). In this regard, Hanjabam *et al*. (2019) reported that the extraction of fucoidan from *Sargassum wightii* using ultrasound method had a higher yield (14.61%) than the hot water method (10.59%). In another study, Okolie *et al*. (2019) compared the fucoidan yield from *Ascophyllum nodosum* using conventional chemical, enzymatic, ultrasound, and microwave extraction methods. Their results showed that the conventional chemical (11.9%) had significantly higher fucoidan yield compared to the other methods (3.89-5.71%).

Recovered fucoidan from *N. zanardinii* by UM method was composed of carbohydrates, proteins, sulfates, and uronic acids. The fucoidans isolated from *Laminaria japonica* using EDTA (0.5% at 70 °C) extraction methods contained 27.95% sulphate, 20.34% uronic acid and 0.86% proteins (Zhao *et al*., 2018). Microwave-extracted fucoidans from *A. nodosum* contained varying levels of sulfate (6.10-29.33%), which differed according to the extraction time and temperature used (Yuan and Macquarrie, 2015).

Extracted fucoidan also was composed of different levels of fucose, galactose, glucose, mannose, and xylose with different molar percentages. This monosaccharide composition was previously reported for fucoidans extracted from *S. polycystum* (Palanisamy *et al.,* 2017), *Sargassum angustifolium* (Borazjani *et al.,* 2018) and *S. glaucescens* (Huang *et al.,* 2016). However, Bahramzadeh *et al*. (2019) reported that the fucoidan from *Cystoseira indica* also possesses Rhamnose in its monosaccharide composition. The fucoidan from *L. japonica* also contains Arabinose further than above-mentioned monosaccharides (Zhao *et al*., 2018).

In the current study, the molecular weight of extracted fucoidans from *N. zanardinii* was 748 kDa. The molecular weight of fucoidans from *L. japonica* and *S. angustifolium* were 64.04 and 421 kDa, respectively (Borazjani *et al.,* 2017; Zhao *et al.,* 2018). The molecular weight of fucoidan from *A. nodosum*, *Fucus vesiculosus*, and *Saccharina longicruris* ranged from 417 to 1323 kDa (Rioux *et al*., 2007). The molecular weight of fucoidans from *L. japonica* by acidic, hot water, and alkaline methods were 60.98, 258.99, and 33.47 kDa, respectively (Sun *et al.,* 2018). Similar to the fucoidan yield, the chemical composition, monosaccharide composition and molecular weight of fucoidans were also affected by algal species, growth conditions, population age, seaweed harvest season and the used extraction technique (Palanisamy *et al.,* 2017; Borazjani *et al.,* 2018).

The cytotoxic effects of *N. zanardinii* fucoidan (62.41 to 78.08% for HeLa cells and from 62.45 to 70.29% for $HepG_2$ cells) were lower than 5-Fu as the positive control. Fucoidans from *L. japonica* and *S. augustifolium* exhibited inhibitory activity of around 30% against HeLa cells (Borazjani *et al.,* 2018; Zhao *et al.,* 2018). Fucoidans of *Undaria Pinnatifida* showed 18-36.5%

inhibitory activity against the Human gastric carcinoma cell line (You *et al.*, 2010). The cytotoxic activity of fucoidans generally depends on seaweed species, growing conditions, harvesting season, extraction, and purification techniques, as well as the cancer cell line being studied (Yang *et al.*, 2008). Furthermore, some inherent features of fucoidan, such as molecular weight, monosaccharide composition, sulphate, content and glycosidic branching, can also have an effect on cytotoxic activity (Zhao *et al.*, 2016). In this regard, previously reported that fucoidans with higher sulfate content displayed higher cytotoxic activity on HeLa cells (Borazjani *et al.*, 2018). Another study reported that the cytotoxic activity of algal polysaccharides might also attribute to the amount of fucose with the positive correlation (Wang *et al.*, 2015).

The current study investigated the effect of UM-extracted fucoidans on the stimulation of RAW264.7 murine macrophage cells. Compared to the control group, proliferations of RAW264.7 cells were significantly increased in the presence of extracted fucoidans. These results suggested that the extracted fucoidan was nontoxic and favorably stimulated the growth of RAW264.7 cells over the concentrations tested. Similar results previously reported by Borazjani *et al*. (2018) for fucoidan extracted from *S. angustifolium*. In another study, Bahramzadeh *et al*. (2019) also reported that the incubation of RAW264.7 cells with fucoidan of *C. indica* samples significantly improved the cell proliferation. The immunostimulatory potential of UM-extracted fucoidan was expressed as the amount of nitric oxide (NO) released from RAW264.7 cells after incubation with different concentrations of fucoidan and it ranged from 35.97 to 37.79 µmol. This value is higher than NO released by RAW246.7 cells treated by *C. indica* fucoidan (less than 20 µmol, Bahramzadeh *et al.*, 2019) and *S. augustifolium* fucoidan (31.7 µmol, Borazjani *et al*., 2018) at the same concentration. Generally, the immunomodulatory activities of polysaccharides depended on their structural characteristics, such as molecular weights, sulfate content, monosaccharide composition, branching degrees, and glycosidic linkages (Ferreira *et al.*, 2015; Zhang *et al.*, 2018). Furthermore, Alboofetileh *et al*. (2019) also reported that the extraction method of fucoidan also can affect its immunomodulatory activity. In that study, the NO productions of RAW264.7 cells in the presence of fucoidans extracted by hot water and different enzymes were 25-42 µmol.

In the current study, the yields, chemical and monosaccharide composition, molecular weight, and biological properties of fucoidan extracted from *N. zanardinii* by UM method were investigated. Extraction yield and molecular weight of fucoidan using this method were 5.53% and 748 kDa, respectively. The fucoidan extracted by UM method contained fucose, galactose, mannose, glucose, and xylose and exhibited the appropriate immunomodulatory and cytotoxic

activity against HeLa and $HepG_2$ cells *in vitro*.

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