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Subcritical water extraction as an efficient technique to isolate biologically-active fucoidans from *Nizamuddinia zanardinii*

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ABSTRACT

A sulfated polysaccharide (fucoidan) has been isolated from *Nizamuddinia zanardinii* using subcritical water ex- traction method (SCWE), and extraction conditions were optimised using the response surface methodology. The optimum extraction conditions were found to be: extraction time of 29 min, extraction temperature of 150 °C, and raw material-to-water ratio of 21 g/mL. The fucoidan yield under these optimum conditions was 25.98%, which was considerably higher than that of conventional solvent extraction (5.2%). Extraction time and temper- ature were the extraction variables that most significantly affected fucoidan yield. Chemical and monosaccharide composition, molecular weight, and the antioxidant, anticancer and immunomodulatory activities of the extract have also been investigated. The monosaccharide composition of fucoidan included fucose (34.13%), mannose (30.70%), galactose (23.19%), xylose (9.35%) and glucose (2.65%). The average molecular weight of the extracted fucoidan was 694 kDa. Antioxidant results revealed that SCWE-extracted fucoidan had appreciable ABTS radical scavenging (70.35%) and reducing power (0.182 Abs). The anticancer activity of fucoidan ranged from 24.60 to 49.46% for HeLa cells and from 23.95 to 46.78% for HepG2 cells. The NO production of RAW264.7 cells was ob- served to be dose-dependent, while maximum NO production was found to be 34.82 μmol at a 50 μg/mL fucoidan concentration.

Keywords:
Nizamuddinia zanardinii Subcritical water extraction Fucoidan, Biological properties, Response surface methodology

1. Introduction

Recent decades have seen the search for novel bioactive compounds from natural resources gaining significant attention because of growing consumer demand for natural products. Seaweed is one of most interesting natural resources for the recovery of bioactive compounds. Seaweed contains a wide range of biological components, including polysaccharides, phenolic compounds, carotenoids, lipids and proteins [1].

Fucoidan, which is extracted from the cell walls of brown seaweed and sea cucumbers, is an anionic sulfated polysaccharide that is mostly made up of fucose, but also contains mannose,

galactose, uronic acids, xylose and sulfated fucose [2]. A wide range of physiological and biological activities has been attributed to fucoidan, including antioxidant, anti-inflammatory, anti-tumour, anti-viral, antidiabetic, anti-obesity, anti-coagulant and antimicrobial properties, among others [3]. These fucoidan bioactivities depend on numerous factors, including molecular weight, sugar composition, sulfate content and position, sulfation degree, the structure of the backbone and branches and the purity of the final product [4–7].

The extraction of fucoidan from brown seaweed is usually accomplished using a conventional extraction technique and either water or aqueous organic solvents [8]. Conventional extraction requires high energy, long extraction times and high experimental costs, but shows low efficiency and extraction yields [9]. It is therefore necessary to develop a method that can overcome these disadvantages.

The subcritical water technique (SCWE, also known as pressurized hot water or hot compressed water) is a modern extraction method that is used for the isolation of high-added-value compounds from raw materials. In SCWE, water is kept under pressure (usually 10/ 60 bar) and heated over his boiling point (up to 374 °C). In such conditions, the physical properties of water (*i.e.* dielectric constant) can be significantly modified. SCWE can make use of variations in temperature to selectively extract polar, bipolar and non-polar compounds from raw materials [10,11]. SCWE has several advantages over conventional extraction methods and these include shorter operation times, higher extraction yields, higher isolated product quality and lower energy consumption [10,12,13]. Furthermore, SCWE is considered to be environmentally and human friendly as it uses water as the only extraction solvent [14,15].

The effectiveness of SCWE depends on several factors, such as extraction time and temperature, the solvent-to-solid-material ratio, and the nature of the compounds to be extracted. Optimising the extraction conditions according to the final products is therefore important for future research, development and application in industry. Response sur- face methodology (RSM) is a statistical experimental design method that is commonly used to optimise the operating parameters of extraction methods [1]. It has been successfully used in the optimisation of the extraction conditions of various added value compounds from natural sources [16].

The SCWE technique has been successfully used in the extraction of many compounds from terrestrial resources. These compounds include polysaccharides [15], phenolic compounds [17], catechin and epicatechin [18], caffeine [19], mengiferin [20], damnacanthal [11], flavonoids, phenolic acids and anthocyanins [21], glycyrrhetic acid, glycyrrhizin and liquiritin [22], isoflavones [23], lignans, proteins and carbohydrates [24] and kava lactones [25]. Moreover, the technique has also been used for the depolymerisation and modification of polysaccharide structure [26,27]. However, to the best of our knowledge, there are only a few re- ports [28] that have investigated the extraction of sulfated polysaccharides from seaweed using the SCWE method.

Previously, we isolated a fucoidan from *Nizamuddinia zanardinii* using conventional hot water extraction and enzyme-assisted extraction. The extraction yields varied between 4.3 and 5.6% which was relatively low considering the high amount of extraction energy, water, time and cost [29]. Therefore, the aims of the present study are: (i) to effectively enhance the extraction yield of fucoidan from *N. zanardinii* (ii) the evaluation of the extracts' structural and physicochemical properties as well as their molecular weight, (iii) investigating the antioxidant, anticancer and immunomodulatory activity of the fucoidan fraction.

2. Material and methods

a. *Algal materials*

The marine brown seaweed, *N. Zarnardinii*, was freshly collected from Chabahr beach in Sistan and Baluchestan province, Iran. The col- lected seaweed was washed with water to remove the non-target materials, attached salt and minerals. The samples were then dried at 40 °C for 72 h, ground using a commercial blender, sieved (b0.5 mm), packed in zip kip plastic bags and stored at −20 °C until use.

b. *Extraction procedure*

2.2.1. Pre-treatment of seaweed samples N. Zarnardinii samples were treated with 85% ethanol (1:10 g/mL) under stirring (2000 rpm) for 24 h at room temperature in order to remove pigments and low molecular weight compounds. Subsequently, the supernatant was removed by vacuum filtration and the residue was recovered. The recovered residue was then washed with acetone and dried overnight at room temperature.

2.2.2. Subcritical water extraction (SCWE) SCWE was performed using Synth wave apparatus (Milestone, Bergamo Italy). In a typical experiment, 10 g of pre-treated seaweed was loaded into the extraction vessel (volume 500 mL) which contained a range of volumes of distilled water across the various experiments, and was heated to the temperature given by the response surface methodology (RSM) software. Before heating, the reactor was purged with nitrogen in order to avoid oxidation during the extraction. During the extraction, a mechanical stirrer (27 cm length and 6 cm head diameter, 425 rpm) was used to improve mass transfer. The independent variables were extraction time (10–30 min), extraction temperature (90–150 °C) and water-to-solid ratio (20–40 mL/g). Other extraction conditions, including desired time for the temperature increase (5 min), pressure (7.5 bar) and power (1500 W) were kept constant. After extraction, the extract was filtered through Whatman filter paper under vacuum (two times), and the supernatant was collected in a glass flask. The supernatant was concentrated under reduced pressure at 60 °C and the concentrated extractwas subsequently mixed with 1% calcium chloride and left overnight at 4 °C to precipitate the alginates.

The alginates were separated using Whatman filter paper and ethanol (99%) was added to the supernatant to achieve a final concentration of 70%. The mixture was maintained overnight at 4 °C and the precipitate was then collected after centrifugation at 4000 rpm for 10 min. The resulting pellet was washed three times with absolute ethanol and twice with acetone. It was then dried at room temperature to give the sulfated polysaccharide (crude fucoidan). The fucoidans were weighted and stored at −20 °C until they were analysed. Fucoidan yields were calculated based on the dried seaweed treated with 85% ethanol and acetone [30].

2.3. Experimental design

RSM, with Box–Behnken design (BBD), was used to determine the optimal conditions for fucoidan extraction from *N. zanardinii*. Three single factors, extraction time (X1), extraction temperature (X2) and water–to-solid ratio (X3), were chosen as the three independent variables.

The experimental ranges for the factors were determined according to our previous research. Fucoidan yields were used as the dependent variables. The experimental design consisted of 17 experimental points, including 12 factorial points and 5 centre points (Table 1), and the experiment was carried out in a random order. The response variables were fitted to a quadratic polynomial model and the general form of the second-order polynomial equation is:

$$Y = \beta 0 + \beta 1\, X1 + \beta 2\, X2 - \beta 3\, X3 + \beta 12\, X1X2 - \beta 13\, X1X3 - \beta 23\, X2X3 + \beta 11\, X1^2 + \beta 22\, X2^2 + \beta 33\, X3^2 \quad (1)$$

where β0 is a constant coefficient of the models. The regression coefficients (β1, β2, and β3), (β11, β22, and β33), and (β12, β13, and β23) represent the linear, quadratic and interaction effects, respectively, of the model, as estimated by multiple regression analysis.

Table 1
Box-Behnken design and results for the yield of fucoidan.

Factor	Unit	Symbols	Level of factor		
			−1	0	1
Extraction time	min	X_1	10	20	30
Extraction temperature	°C	X_3	90	120	150
Ratio of water to raw material	mL>/g	X_4	20	30	40
Std. order	X_1	X_2	X_3	Yield (%)	
1	−1	−1	0	4.99	
2	1	−1	0	6.36	
3	−1	1	0	19.4	
4	1	1	0	22.47	
5	−1	0	−1	7.75	
6	1	0	−1	14.42	
7	−1	0	1	10.12	
8	1	0	1	11.95	
9	0	−1	−1	5.94	
10	0	1	−1	23.77	
11	0	−1	1	6.38	
12	0	1	1	21.25	
13	0	0	0	9.22	
14	0	0	0	9.55	
15	0	0	0	8.95	
16	0	0	0	7.73	
17	0	0	0	8.78	

2.4. Fourier-transform infrared spectroscopy analysis (FT-IR)

The fucoidan samples were mixed with potassium bromide and powdered using an agate mortar. The powders were loaded into the testing cell and the FT-IR spectra of the samples were collected in the 400–4000 cm^{-1} range using a Fourier transform IR spectrophotometer (Bruker Instruments, Billerica, USA).

2.5. Chemical composition

Total carbohydrate content in extracted fucoidan was determined using the phenol–sulfuric acid method with D-fucose as the standard [31]. The Lowry method was used to estimate protein impurities with bovine serum albumin as the standard [32]. Sulfate content was determined using the $BaCl_2$ gelatin method [33]. Uronic acid content was quantified using the *m*-hydroxybiphenyl method with D-glucuronic acid the standard [34].

2.6. Determination of monosaccharide composition

Gas chromatography mass spectrometry (GC–MS) was used to determine the monosaccharide composition of extracted fucoidan. Sample preparation was performed according to a procedure that was previously eported by Tabarsa et al. [35]. The GC–MS system consisted of an Agilent 7890 N instrument equipped with a HP-5 capillary column (30 m × 0.32mm× 0.25 μm) and a flame ionization detector. The temperature program functioned as follows: oven temperature was initially set at 120 °C, then increased to 240 °C at a rate of 10 °C/min and was then held at 240 °C for 6min. The heater

temperatures of both the injector and detector were kept at 250 °C. Nitrogen was used as the carrier gas. The results were reported as the relative peak areas. Fucose, rhamnose, xylose, mannose, galactose, arabinose and glucose were used as monosaccharide standards, according to the reference.

2.7. 2.7. Determination of molecular weight

A HPSEC–UV–MALLS–RI system (high-performance size exclusion chromatography column coupled to UV, multi-angle laser light scattering, and refractive index detection) was used to determine themolecular weight of the sulfated polysaccharides. Sample preparation was performed according to a procedure that was previously reported by Anvari et al. [36]. The average molecular weight (Mw) of fucoidan was calculated using ASTRA 5.3 software (Wyatt Technology Corp.).

2.8. Scanning electron microscopy (SEM)

The SCWE-extracted polysaccharides were mounted on a copper holder with aluminium tape and coated with thin layer of gold, using a BAL-TEC SCD 005 sputter coater (BAL-TEC AG, Balzers, Liechtenstein) under reduced pressure. The prepared samples were observed using a Philips XL 30 scanning electron microscope (Philips, Eindhoven, Netherlands) at an accelerating voltage of 20.0 kV.

2.9. Surface colour measurement

The colour of the SCWE-extracted fucoidan was measured using a colour meter (BYK Gardner, USA) and expressed as L* (lightness), a* (red/green), and b* (yellow/blue) values. The colour differences (ΔE) and whiteness indices (WI) of the samples were calculated with respect to standard plate parameters (L*=94.63, a*= −0.88, and b*=0.65) using the following equations:

$$\Delta E = \sqrt{(\Delta a^*)^2 + (\Delta b^*)^2 + (\Delta L^*)^2} \quad (2)$$

$$WI = 100 - \sqrt{(100 - L^*)^2 + a*^2 + b*^2} \quad (3)$$

2.10. Determination of antioxidant activity

2.10.1. ABTS scavenging activity

The assay was carried out according to a method developed by Borazjani et al. [5]. Briefly, serial dilutions of the extracted fucoidan at different concentrations (0.125–1 mg/mL) were prepared in distilled water. The ABTS radical cation was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulfate in dark at room temperature for 16 h. After this time, the ABTS radical cation solution was diluted with ethanol to an absorbance of 0.70 at 734nm. To determine the scavenging activity, 50 µL of fucoidan samples solutionwas taken and transferred into 96-well microplates. Next, 150 µL of ABTS solution wasadded to polysaccharide samples and then, the reaction mixture was incubated for 20 min in the dark at room temperature. Finally, the absorbance of samples solution was measured at 734 nm using ELISA microplate reader. Ascorbic acid (100 µg/mL) was used as positive control.

The following equation was used for calculating the ABTS radical scavenging activity:

ABTS scavenging activity (%)= (Ac–As/Ac)x100 (4)

Ac: the absorbance of control (50 μL of ethanol with 150 μL of the ABTS solution)
As: the absorbance of polysaccharide sample solution

2.10.2. Reducing power

200 μL of fucoidan samples with different concentrations (0.125–1 mg/mL) were mixed with 500 μL of phosphate buffer (0.2 M, pH 6.6) and 500 μL of potassium ferricyanide (1%) in 2 mL micro tube. After incubation at 52 °C for 30 min in water bath, 500 μL of 10% TCA was added to the tubes and the mixture was centrifuged (10,000 rpm, 10 min). After centrifugation, 500 μL of supernatant transferred into new micro tube. Next, 500 μL of distilled water and 100 μL of ferric chloride (Fe^{3+}, 0.1%) were added to the tubes and then, the reaction mixture was incubated for 10min at room temperature. Finally, the absorbance of samples solution was measured at 700 nm using ELISA microplate reader. Ascorbic acid (100 μg/mL) was used as positive control [37].

2.11. Determination of anticancer activity

Human epithelioid cervix carcinoma (HeLa) and human hepatocyte carcinoma (HepG2) cell lines were used to evaluate the anticancer activityof SCWE-extracted polysaccharides. Cancer cells were plated in 96-well plates and incubated for 4 h at 37 °C with 5% CO_2 saturation.

After incubation, the cell cultures were treated with various concentrations of either SCWE-extracted fucoidan (100, 200 and 400 μg/mL) or 5-fluorouracil as positive control (10 μg/mL, 5-Fu, Sigma, St. Louis, MO, USA) and incubated at 37 °C for 72 h. At the end of the incubation, the anticancer activity of the sulfated polysaccharides was determined using the WST-1 colorimetric assay kit (Roche Diagnostics, Madison, WI, USA) under previously reported conditions [38].

2.12. Determination of macrophage proliferation activity and nitric oxide releasing capacity

RAW264.7 cells were seeded in 96-well microplates at a density of 1 × 104 cells per well. The medium was RPMI-1640 that had been supplemented with 10% FBS and the cells were grown at 37 °C with 5% CO_2 saturation. The cell cultures were then treated with various concentrations of SCWE-extracted fucoidan (10, 25, 50 μg/mL) and the microplates were incubated at 37 °C in 5% CO_2. The absorbance of each well was measured at 450 nm using a microplate reader (EL-800; BioTek Instruments, Winooski, VT, USA). Nitric oxide (NO) production in the RAW264.7 cell culture supernatant was measured and used as an indicator of the immune-enhancing activity of the polysaccharides. The concentration of NO was measured according to the colorimetric Griess reaction, as described by Green et al. [39].

2.13. Statistical analyses

Design-Expert software (version 7.1.6) and SPSS (version 16.0) statistical softwarewere used to statistically analyse the data. Data are presented as mean values ± SD. One way ANOVA and Duncan's test (p b 0.05) were performed to calculate the differences between the concentrations of the polysaccharides in different tests.

3. Results and discussion

3.1. Optimization of fucoidan extraction by BBD

3.1.1. Model fitting and statistical analyses

There were a total of 17 runs for the optimisation of the three individual parameters in the BBD. Table 1 presents the extraction yield of fucoidan (dependent variables) for each run in the experimental design. The fucoidan yield from *N. zanardinii* ranged from 4.99 to 23.77%. Experiment 10 gave the maximum yield with following features:extraction time 20 min, extraction temperature 150 °C and water-to-material ratio 20 mL/g. Experiment 1 gave the lowest yield with following features: extraction time 10 min, extraction temperature 90 °C and water-to-material ratio 30 mL/g. The data underwent multiple regression analysis in Design-Expert 7.1.6 and the following second-order polynomial equation was derived to represent fucoidan yield as a function of the independent variables tested. The causal relationship between the response variable (fucoidan yield) and the three test variables is described in the equation:

$$Y = 8.77 + 1.62X1 + 7.90X2 - 0.27X3 + 0.42X1X2 - 1.21X1X3 - 0.74\ X2X3 + 0.63\ X1^{2} + 3.90X2^{2} + 1.66X3^{2} \quad (5)$$

where Y represents the yield of fucoidan, X1, X2 and X3 are the coded values of the test variables for extraction time, extraction temperature and water-to-material ratio, respectively.

Table 2
Analysis of variance for the fitted quadratic polynomialmodel of extraction of fucoidan. a, b

Source	Sum of squares	DF	Mean square	*F* Value	*p*-Value
Model	612.41	9	68.05	80.08	<0.0001[a]
X_1	20.93	1	20.93	24.63	0.0016[a]
X_2	499.60	1	499.60	587.97	<0.0001[a]
X_3	0.59	1	0.59	0.70	0.4307[b]
$X_1 X_2$	0.72	1	0.72	0.85	0.3871[b]
$X_1 X_3$	5.86	1	5.86	6.89	<0.0341[a]
$X_2 X_3$	2.19	1	2.19	2.58	0.1524[b]
X_1^2	1.66	1	1.66	1.95	0.2048[b]
X_2^2	64.14	1	64.14	75.49	<0.0001[a]
X_3^2	11.57	1	11.57	13.62	<0.0077[a]
Residual	5.95	7	0.85		
Lack of fit	3.14	3	1.05	1.50	0.3438[b]
Pure error	2.80	4	0.70		
Cor total	618.36	16			
R^2	0.9904				
Adj. R^2	0.9780				
Pred. R^2	0.9116				
Adeq. precision	26.931				
CV%	7.89				

DF Degrees of freedom.
[a] Significant ($p < 0.05$).
[b] Not significant ($p > 0.05$).

The ANOVA analyses for the fitted quadratic polynomial model of fucoidan yield are shown in Table 2. According to the results, the quadratic regression model had a high F-value (F= 80.08) and a very low P-value (P b 0.0001), indicating that the fitness of the model was highly significant. F-value for the lack of fit of this study was insignificant (1.50) for selected variables in themodel, thereby

confirming the validity of the model. The goodness-of-fit of the regression model was carried out by determination coefficient (R^2) and adjusted determination coefficient (R^2adj). The high values of R^2 (0.9904), adjusted R^2 (0.9780) and Pred R-Squared (0.9116) were reasonably close to 1, indicating a high degree of correlation between the experimental and predicted values. Furthermore, the low values of coefficient variation (C.V. %) and the high values of Adeq. precision were 7.89 and 26.931, respectively, and indicate a high degree of precision and reliability in the experimental values.
The significance of each coefficientwas checked using the P-value. In this case, the variable with the lowest P-value will be more significant. In our current study, the linear coefficients (X1 and X2), quadratic coefficients ($X2^2$ and $X3^2$), and cross-product coefficients (X1X3) had a significant effect, with small p-values (p b 0.05).

3.1.2. Optimisation of Fucoidan extraction conditions

RSM and BBD were used to optimise the extraction conditions and maximize the isolation of fucoidan from *N. zanardinii*. Fig. 1 shows the three-dimensional (3D) response surface plots. This figure presents the relationship between extraction variables (extraction time, extraction temperature and water-to-material ratio) and fucoidan yield as well as changes in extraction yield under different conditions. Theplots were plotted by selecting two independent values and keeping the other at the zero level. Fig. 1A shows the effect of extraction time (X1), extraction temperature (X2), and their interaction on fucoidan yield. The effect of extraction time and extraction temperature on yield revealed that the recovery of fucoidan increased when the two variables were increased. Fig. 1B shows the relationship between polysaccharide yields and two variables, extraction time (X1) andwater-tomaterial ratio (X3). Results show that polysaccharide yield values increased with increasing extraction time, from 10 to 30 min, while yield decreased with increasing water-to-material ratio, from 20 to 30 mL/g, but then increased with further increases in water-to material ratio, up to 40 mL/g. Fig. 1C indicates that extraction temperature (X2) and water-to-material ratio (X3) have a mutual effect on fucoidan yield. Results show that polysaccharide yield increased with increasing extraction temperature, whereas increasing the water-to material ratio resulted in a slight increase in polysaccharide yield.

3.1.3. Validation of the model

In this study, Design-Expert software suggested the optimal values for tested independent variables giving the following response variables for the proposed extraction: extraction time of 28.64 min, extraction temperature of 149.69 °C, water-to-material ratio of 20.92 mL/g, and a maximum predicted extraction yield of 25.87%. In order to validate the adequacy of themodel equation, a verification experimentwas carried out under the modified optimal conditions: extraction time of 9 min, extraction temperature of 150 °C and water-to-material ratio of 21 mL/g. Triplicate confirmatory experiments were carried out under these conditions and the average fucoidan yield was 25.98%, which was very close to the corresponding predicted value. This value is considerably higher than that of conventional solvent extraction of fucoidan from *N. zanardinii* (5.2%) [29]. Furthermore, this extraction yield is higher than the fucoidan content of *Arthrothamnus bifidus* (0.4%), *Agarum cribrosum* (1.2%), *Laminaria longipes* (2.4%), *Sargassum binderi* (6.16%), *Ascophyllum nodosum* (11.3%), *Ascophyllum nodosum* (6.48–16.08%), and *Saudersella simplex* (20.4%) samples [40–42]. These discrepancies in the yields of fucoidan from the different brown seaweeds are believed to have

originated from the differences in the species, collection season and location of the seaweed, as well as the type of extraction method [6,41,43].

3.2. Infrared spectroscopy analyses

The FT-IR spectra of fucoidan isolated from N. zanardinii by SCWE method was shown in Fig. 2A. As can be seen, different absorption signals were recorded in the range of 400–4000 cm−1 for UAE-extracted fucoidan. The infrared spectra in the two regions of 1200–800 and 3400 cm^{-1} region was in accordance with the characteristic wavenumbers of polysaccharides. Signal at 818 cm^{-1} was attributed to the bending vibrations of C\\O\\S of sulfate and it indicated a complex substitution of 4-sulfate and 6-sulfate monosaccharide units [44].

The signal at 1000–1010 cm^{-1} was attributed to glycosidic links [45]. The peak near 1250 cm^{-1} was attributed to primary and secondary O sulfate groups, which is a characteristic component of fucoidan and sulfated polysaccharides in marine seaweeds [46]. The peaks at 1420 cm^{-1} and 1620 cm^{-1} indicate the presence of the symmetrical bending vibration of CH_3, and asymmetrical bending vibration of CH_3, respectively [41].Weak signals around 2930 cm^{-1} resulted fromC-6 group of fucose, galactose or C\\H stretching vibrations of carbohydrates. Broad signal at 3424 cm^{-1} represents the O\\H stretching of hydroxyls common to all polysaccharides [47]. The finding is that the peaks of fucoidan extracted in this study are very similar to those of the peaks reported for fucoidan extracted from *S. glaucescens*, *S. polycystum*, and *S. binderi* [41,47,48].

3.3. Chemical profile analysis

The carbohydrate, protein, uronic acid and sulfate contents of extracted fucoidan were 50.49 ± 0.74%, 5.14 ± 0.37%, 2.07 ± 0.12% and 10.34±0.59%, respectively. Hot water extracted fucoidan that was isolated from *Sargassum polycystum* contained 38.76 ± 0.26% carbohydrate, 22.35 ± 0.23% sulfate, 3.9 ± 1.8% uronic acid and 4.7 ± 0.43% protein [47]. As can be seen, the sulfate content of SCWE-extracted fucoidan is in the wide 3.6–51.3% range, as reported for fucoidans from other brown seaweeds [49]. The sulfate content of SCWE extracted fucoidan (10.34%) is higher than reported values for fucoidan that was isolated from *Padina gymnospora* (5.7%), and *Padina tetrastromatica* (3.6–19%), but lower than those reported for fucoidan from *Ascophyllum nodosum* (19–22%), and *Turbinaria tricostata* (19.5–22.7%) [42,49]. Higher polysaccharide sulfate content is an advantageous property as it has been reported that polysaccharides with higher sulfate content display higher biological activity, including antioxidant, antitumour, antiviral and anticoagulant properties, among others [42]. SCWE-extracted fucoidan is composed of fucose (34.13 ± 0.54%), mannose (30.70 ± 0.54%), galactose (23.19 ± 0.24%), glucose (2.65 ± 0.05%), and xylose (9.35 ± 0.19%). Rhamnose and arabinose were not found in the extracted fucoidan (Fig. 2B). Sulfated polysaccharides that were extracted from *Sargassum polycystum, Sargassum augustifolium, Sargassum glaucescens* and *Laminaria japonica* were also found to be composed of fucose, galactose, glucose, mannose and xylose [47,48,50,51]. The chemical and monosaccharide composition of fucoidans can be affected by algal species, the age of the population, environmental conditions, geographic location, harvest season and extraction technique [47].

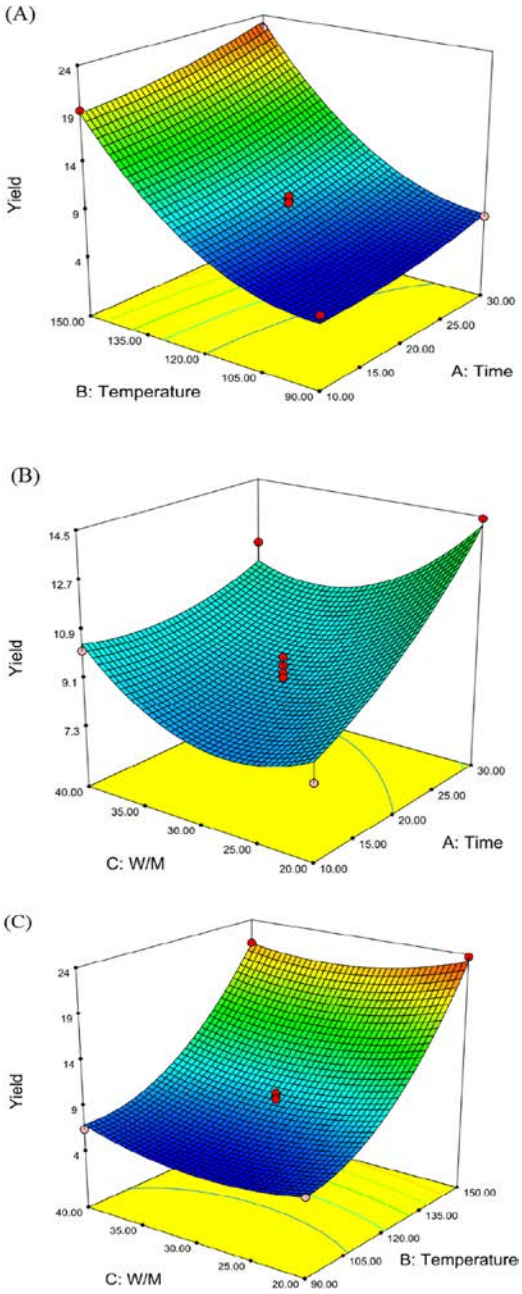


Fig. 1. Response surface plots (A–C) showing the effects of extraction time; extraction temperature; and water-to-raw material ratio on the fucoidan yield.

3.4. Molecular characteristics

Fig. 2C shows the superimposed RI chromatograms for SCWE-extracted fucoidan. As shown in the RI chromatogram, extracted fucoidan had one major peak at the elution time of 45 min. The peak at the elution time of 66 min is related to calcium chloride. As shown in Fig. 2D, SCWE-extracted fucoidan showed a strong UV peak at the elution time of 66 min, which confirmed the presence of protein in the isolated polysaccharide matrix. This result is in accordance with the protein content of extracted fucoidan. The weight average molecular weight (Mw) of extracted fucoidan was 694 ± 3.82 kDa. Anastyuk et al. [52] reported that the average Mw of fucoidan that was isolated from *Saccharina cichorioides*, using an acidic extraction method, was 18–30 kDa. The average Mw of fucoidan fractions that was acid extracted from the sporophyll of *Undaria Pinnatifidawas* 1.6–262 kDa [53].

The average Mw of fucoidan that was microwave extracted from *Ascophyllum nodosum*, collected in the UK (Bod Ayre Products Ltd., Shetland), was 1.34–37.54 kDa [42]. It has been reported that the molecular weight of polysaccharides can be affected by extraction and purification techniques, algal species and growing conditions [50].

3.5. Morphological analyses

The surface morphology of SCWE-extracted fucoidan was observed using scanning electronmicrographs (SEM) and photographs atmagnifications of 200, 500 and 1000, which are illustrated in Fig. 3. The SEM micrographs of SCWE-extracted fucoidan showed rough irregular particles of different sizes. Polysaccharides that were extracted from litchi pulp using hot water have been observed to exhibit a porous structure [54]. Polysaccharides extracted from *Laminaria japonica* using acidic,hot water and alkaline extraction methods display irregular, rough fragments, a smooth sheet-like appearance and rough, irregular particles, respectively [55]. The SEM of the polysaccharide extracted from *Lentinus edodes* using hot water, and an enzyme-microwave ultrasound assisted extraction method have been observed to exhibit a spongy structure, and intact and smooth surface with a few holes, respectively [56]. These findings suggest that the difference in the shape and surface morphology of an extracted polysaccharidemight originate from differences in extraction and purification methods and/or the preparation of the product [56,57].

3.6. Optical analyses

Bioactive polysaccharides such as fucoidan could be incorporated infood systems in order to produce functional foods. The incorporation of fucoidans in food systems in case their colors are dark, makes a considerable impact on the final colour of the product which can be unfavorable.

On this basis, we measured the colour of fucoidan to understand the potential usage of prepared fucoidan in food systems. The values of L*, a*, and b* for extracted polysaccharides were 24.84, 8.37 and 4.47, respectively. Previously, Sun et al. [55] reported the colour of polysaccharides from Laminaria japonica by acidic, water and alkaline extraction methods. They found that the polysaccharide extracted by acidic (86.39) method had higher L* value than water (68.7) and alkaline (66.20) methods. Furthermore, higher yellowness (11.99) and lower redness (0.79) valueswere observed for acid extracted polysaccharides.

The differences in colour of polysaccharides might be related to the different extraction process. The difference in the colour of extracted polysaccharide might have originated from differences in extraction and purification methods. The degree of total colour difference from the standard colour plate (ΔE), and whiteness index (WI) were also investigated, giving values of 70.50 and 24.25, respectively. As can be seen, the polysaccharide has a low value for lightness (L*) and WI. Furthermore, the polysaccharide colour gave a high value for total colour difference from the standard colour plate. This result can be explained by the presence of pigments in the final polysaccharide product.

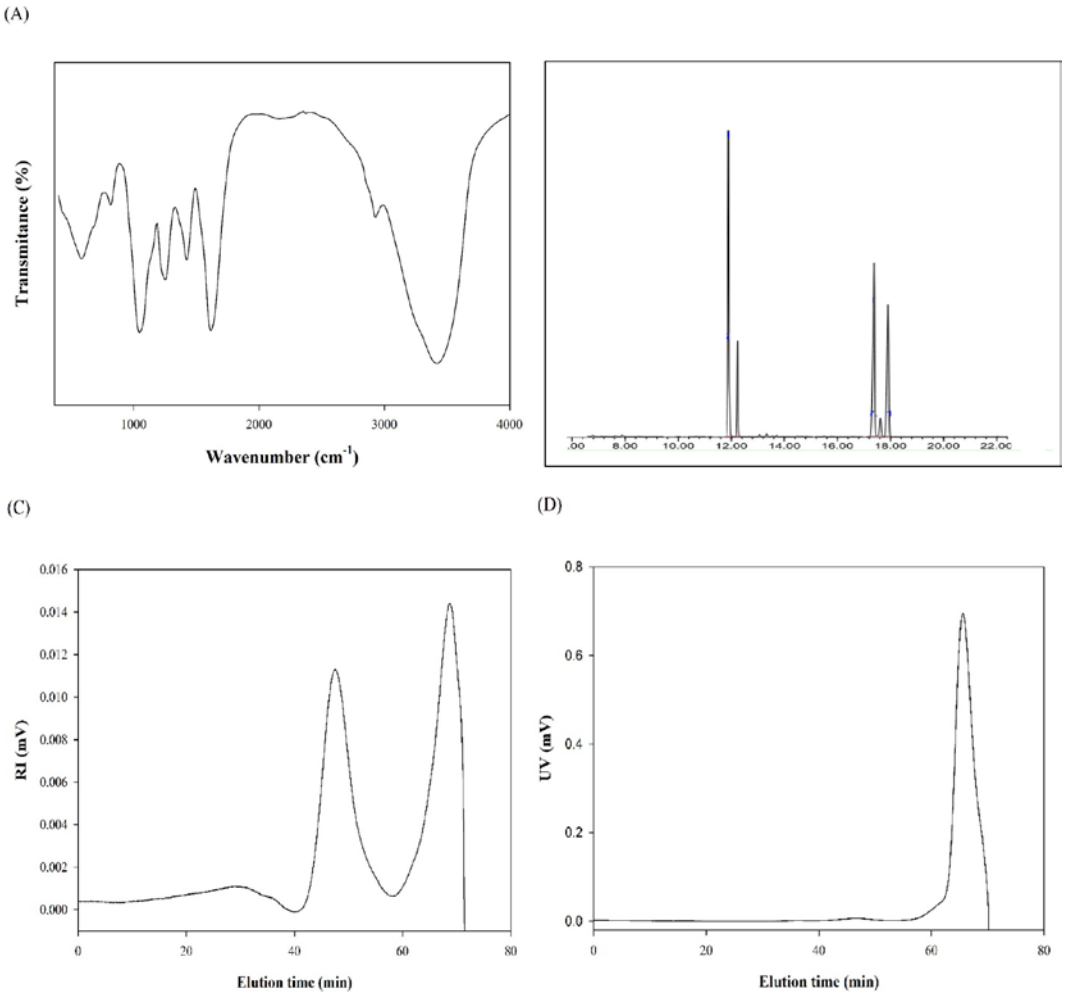


Fig. 2. FT-IR spectra (A); GC–MS chromatograms (B); RI (C); and UV chromatograms (D) of SCWE-extracted fucoidan.

3.7. Antioxidant activity

3.7.1. ABTS radical scavenging activity

The ABTS scavenging effects of SCWE-extracted fucoidan was evaluated at concentrations ranging from 0.250 to 1 μg/mL. The extracted fucoidan showed diverse ABTS scavenging activities (32.31–70.35%), which it significantly increased with increasing the concentrations of fucoidan (Fig. 4A). Borazjani et al. [5] have previously reported that hot water-extracted fucoidan from *Sargassum polycystum* displayed 64% ABTS scavenging activity at 250 μg/mL. In another study, Cui et al. [58] reported that polysaccharides isolated from *Laminaria japonica* possessed about 25% ABTS scavenging activity at 4 mg/mL.

Sulfate content and themolar ratio of sulfate content to fucose aswell as themolecular weight could influence the antiradical activity of the extracts [59]. In this regards, sulfated polysaccharides with higher molecular weights and sulfate contents exhibit higher antioxidant activities [60]. SCWE-extracted fucoidan had higher molecular weight and sulfate content than those extracted from *L. japonica*.

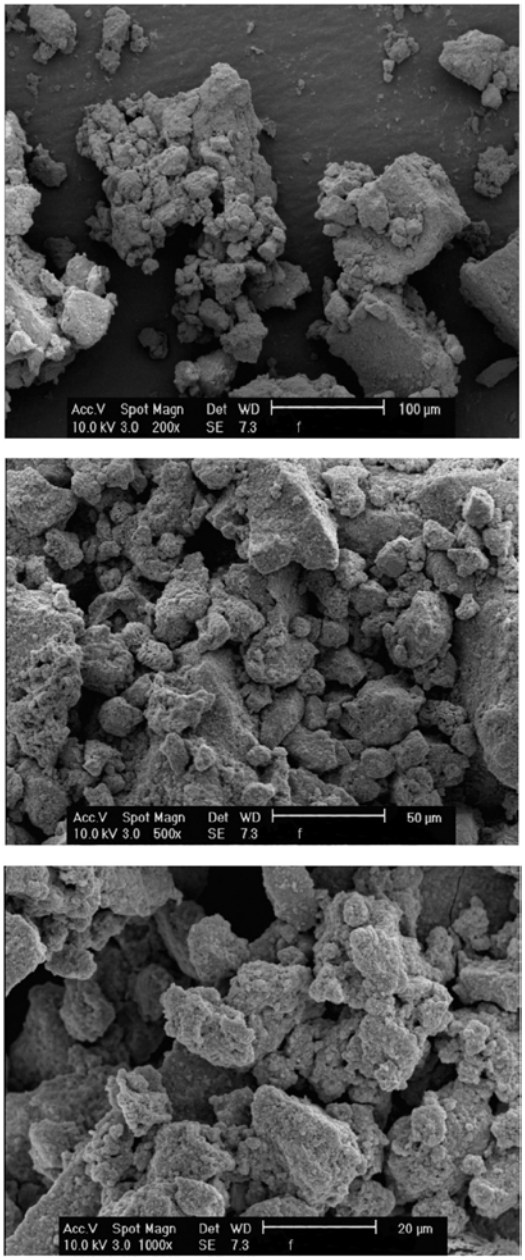
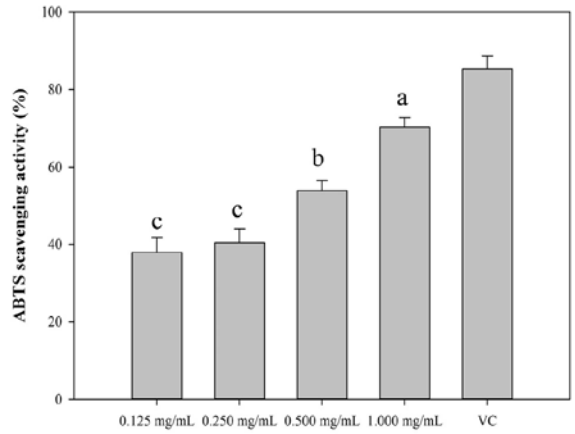


Fig. 3. Scanning electron micrographs of SCWE-extracted fucoidan at different magnifications.

3.7.2. Reducing power

Generally, there is a direct and positive correlation between reducing power and antioxidant activity of samples. Moreover, the reducing power is associated with the presence of reductones in the tested samples. The reducing power of SCWE-extracted fucoidans was evaluated at concentrations ranging from0.250 to 1 µg/mLand the results are shown in Fig. 4B. Higher absorbance value means stronger reducing power of samples. As can be seen, reducing power ability of SCWE-extracted fucoidan increased with increasing sample concentration. Maximum reducing power (0.182 Abs) were obtained in 1 mg/mL, which were weaker than those of ascorbic acid (0.345 Abs). The reducing power of SCWE-extracted fucoidan from *N. zanardinii* is lower than those of native fucoidan from *S. angustifolium* at same concentration (1 mg/mL) and those of fucoidan from *S. glaucescens* ranging from 0.45 to 0.7 at 2 mg/mL [5,48].

(A)



(B)

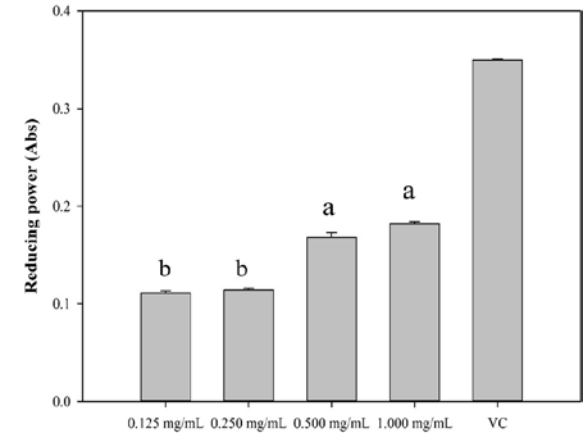
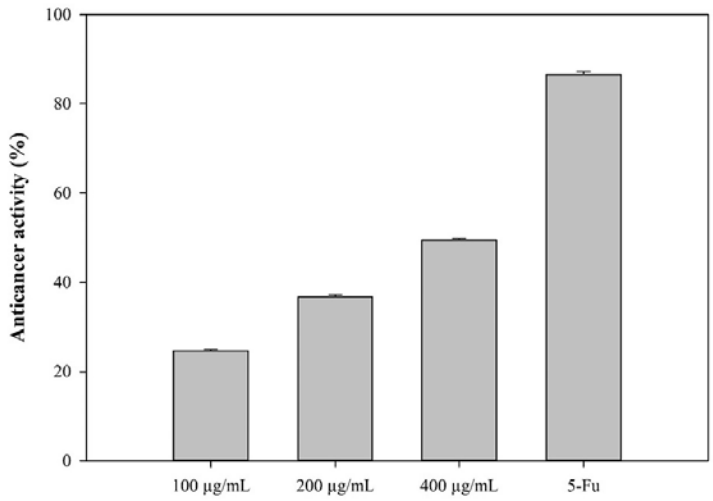


Fig. 4. ABTS radical scavenging (A) and reducing power (B) of SCWE-extracted fucoidan (n=3, means ± SD). The letters a, b, c indicate a significant difference (p b 0.05) between the concentrations of the fucoidan.

(A)



(B)

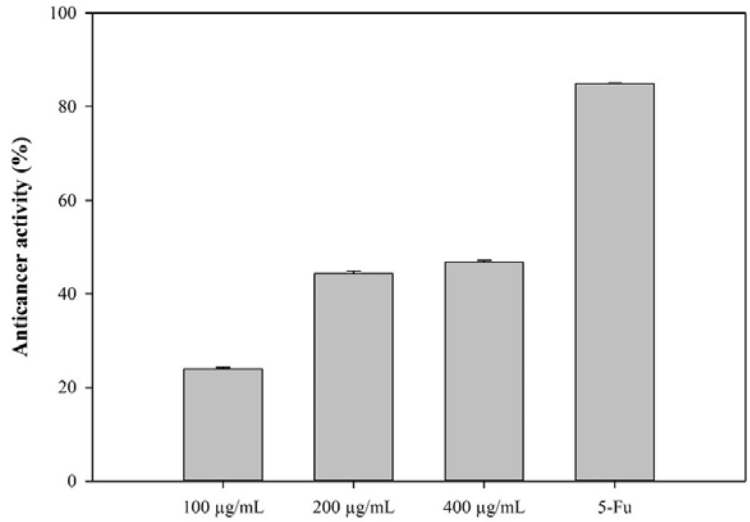
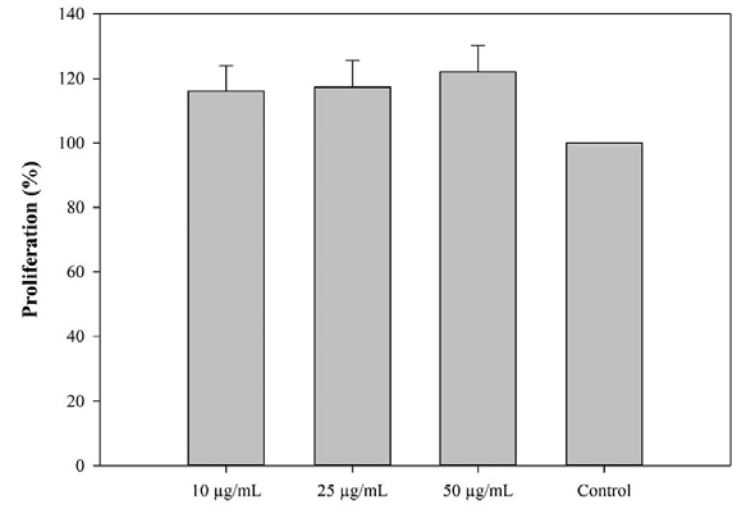


Fig. 5. Effects of SCWE-extracted fucoidan on proliferation of HeLa (A) and HepG2 (B) cancer cells compared with controls (n= 3, means ± SD). The letters a, b, c indicatea significant difference (p b 0.05) between the concentrations of the fucoidan.

(A)



(B)

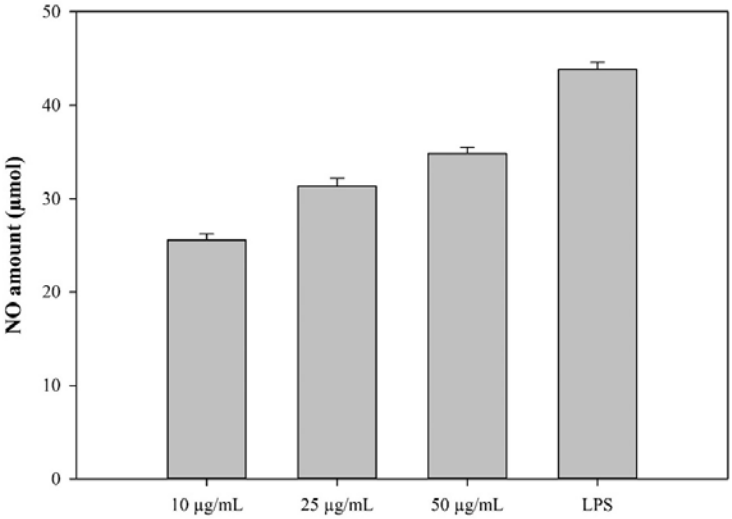


Fig. 6. Effects of SCWE-extracted fucoidan on proliferation (A) and NO production (B) in RAW264.7 macrophage cells (n = 3, means ± SD). The letters a, b, c indicate a significant difference (p b 0.05) between the concentrations of the fucoidan.

3.8. Anticancer activity

Generally, fucoidans can directly kill cancer cells through apoptosis, antiangiogenesis, inhibiting the cellular migration and enhancing various immune responses [61]. Until now fucoidans showed antiproliferative activities on different cancer cell lines such as A549, MCF-7, MDAMB- 231, PC-3 and HCT-15 cell lines [3]. This study also evaluated the in vitro anticancer potential of SCWE-extracted fucoidan against the HeLa and HepG2 cell lines, at concentrations of 100, 200 and 400 µg/mL. The anticancer activity of fucoidan ranged from 24.60 to 49.46% for HeLa cells and from 23.95 to 46.78% for HepG2 cells (Fig. 5). It was observed that extracted fucoidan showed slightly higher anticancer activity against HeLa than against HepG2 cells. Fattah et al. [62] have previously reported that HepG2 cells are more sensitive to a polysaccharide extracted from Bacillus subtilis

NRC1aza than HeLa cells. SCWE-extracted fucoidan from *N. zanardinii* demonstrated higher anticancer activity than was reported for fucoidans from *Laminaria japonica* and *Sargassum augustifolium*, which exhibited inhibitory activity of around 30% against HeLa cells [50,63]. However, the anticancer effect of *N. zanardinii* fucoidan was lower than values that have previously been reported for *Sargassum polycystum* fucoidan, which exhibited 50% inhibitory activity against MCF-7 cells at 50 μg/mL [47]. The anticancer 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 [30]. Furthermore, some inherent features of fucoidan, such asmolecularweight,monosaccharide composition, sulfate content and glycosidic branching, can also have an effect on anticancer activity [64]. Borazjani et al. [50] reported that fucoidans with higher sulfate content displayed the higher anticancer activity on HeLa cells. The presence of sulfated groups in SCWE-extracted fucoidan was confirmed by FT-IR analysis (Fig. 2A) and determination of sulfate content (Section 3.3). A previous study suggested that the higher cytotoxic activity of algal polysaccharides might also attribute to the higher amount of fucose [65]. Our data showed that the fucose content of SCWE-extracted fucoidan was 34.13%.

3.9. Immunomodulatory activity

The immunomodulatory activity of SCWE-extracted fucoidan on RAW 264.7 cells is presented in Fig. 6. As can be seen in Fig. 6A, SCWE-extracted fucoidanwas not cytotoxic to RAW264.7 cells at tested concentrations. Furthermore, it led to increased macrophage cell proliferation compared to the control group. It has been reported that mitogen-activated protein kinases (MAPKs) have an important role to play in cell proliferation and polysaccharides can activate this enzyme [66]. The increases in macrophage proliferation in the present study may thus be related to the activation of MAPKs by the SCWE-extracted polysaccharide.

The effects of SCWE-extracted fucoidan on theNO production of murine macrophage RAW264.7 at tested concentrations (10, 25, 50 μg/mL) are shown in Fig. 6B. The results showthat NO production by RAW264.7 cells increasedwith increasing SCWE-extracted fucoidan concentration.

NO secretion by RAW246.7 cells that had been treated with SCWE-extracted polysaccharide, at 10, 25 and 50 μg/mL, was 25.56, 31.34 and 34.82 μmol, respectively. Borazjani et al. [50] have previously reported that NO production by RAW264.7 cells that had been treated with *Sargassum augustifolium* polysaccharides, at 50 μg/mL, was 31.7 μmol. The difference in the immunomodulatory activity power of polysaccharides may be related to the their structural features, such as monosaccharide composition, sulfate content, functional groups, molecular weight, branching degrees and glycosidic linkages [67].

4. Conclusion

The present study provides a comprehensive evaluation of the chemical profile, average molecular weight, aspects of morphology, anticancer and immunomodulatory activity of fucoidan that has been isolated from *N. zanardinii* using SCWE. RSM and BBD were used to optimise the extraction conditions. The optimal extraction conditions were: extraction time of 29 min, extraction temperature of 150 °C and a raw water-to-material ratio of 21 mL/g. Under these conditions, the fucoidan yield was 25.98%, which is in agreement with the predicted value (25.87%). Fucose, galactose, mannose, glucose and xylose are the monosaccharides present in SCWE-extracted fucoidan. Isolated fucoidan

exhibited the appropriate antioxidant, immunomodulatory and anticancer activity against HeLa and HepG2 cells in vitro.

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