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Effect of different non-conventional extraction methods on the antibacterial and antiviral activity of fucoidans extracted from *Nizamuddinia zanardinii*

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Abstract

In the current study, fucoidans from brown alga *Nizamuddinia zanardinii* were isolated with conventional and non-conventional extraction procedures to evaluate the effects of recently introduced technologies on biochemical characteristics and saccharide composition of the extracts, along with their antibacterial, antiviral and cytotoxic properties. The results demonstrated that subcritical water extraction showed the highest fucoidans yield (13.15%), while the lowest yield was obtained using viscozyme extraction method (4.28%). The polysaccharide chains consisted of fucose, galactose, glucose, mannose and xylose, whose molar percentages differed according to the extraction method used. The weight mean average molecular weight of fucoidans varied between 444-1184 kDa. The FT-IR spectroscopy confirmed the presence of sulfate esters by bending vibration of C–O–S and stretching vibration of S=O peaks at 818 and 1250 cm^{-1}, respectively. Antibacterial assays showed that microwave- and subcritical water-extracted fucoidans inhibited the growth of *E.coli* and that enzyme-ultrasound, ultrasound-microwave and subcritical water extracted fucoidans exhibited inhibitory effects against *P. aeruginosa* at 2 mg/mL. Antiviral studies revealed that all the extracted fucoidans exerted strong antiviral activity against HSV-2 infection, with EC_{50} values in the 0.027-0.123 μg/mL range; indeed the viscozyme-extracted macromolecules displayed the best selectivity index.

Keywords: *Nizamuddinia zanardinii*, Fucoidans, Non-conventional techniques, Extraction methods, Antibacterial activity, Antiviral activity

1. Introduction

Marine seaweeds contain several polysaccharide types, including fucoidans, laminarin, carrageenan, ulvan, agar and alginate among others. Seaweed polysaccharides, especially fucoidans and laminarin, have attracted attention as they show a wide range of therapeutic properties and relatively low toxicity [1]. Previously published reports have indicated that fucoidans possesses several biological activities, including antioxidant, anti-inflammatory, anti-tumor, anti-viral, anti-diabetic, anti-obesity, anti-coagulant and antimicrobial actions [2-9].

The choice of a suitable extraction method is a crucial step in the recovery of polysaccharides from raw material, as this decision may affect the yield, composition, structure and integrity of the desired bioactive polysaccharides [10]. Polysaccharides are typically isolated by either conventional maceration or percolation in either hot water or organic solvents [11]. However, these extraction methods display poor efficiency and have a high environmental impact [12] owing to large solvent amounts, prolonged heating and long extraction time [13], which can damage the polysaccharide structures [14]. Recent decades have seen the development of a number of non-conventional extraction techniques, including the use of enzymes and non-conventional energy sources (microwave, ultrasound, subcritical water and supercritical fluid). Furthermore, these innovative extraction methods have the capacity to modify the chemical composition, molecular properties and biological activity of the target polysaccharides [15].

While a number of papers have highlighted the antiviral and antibacterial activity of polysaccharides extracted from marine seaweed species, the antiviral and antibacterial activities of sulfated polysaccharides from *Nizamuddinia zanardinii (N. zanardinii)* have not been reported yet. Moreover, in the previous reports the marine polysaccharides were extracted using conventional methods and the effects of several non-conventional extraction techniques on chemical, molecular and biological activities of marine polysaccharides have to be explored comprehensively yet.

The current study investigated nine different non-conventional extraction methods, i.e. alcalase, flavourzyme, cellulase, viscozyme, ultrasound, microwaves, alcalase-ultrasound, microwave-ultrasound, and subcritical water, in addition to conventional methods (hot water), for their ability to extract sulfated

80 polysaccharides from *N. zanardinii*. The influence of these extraction methods on the yield, chemical
81 profile, monosaccharide composition and primary structural characteristics as well as on the antibacterial,
82 antiviral and cytotoxic activities of the recovered polysaccharides have been investigated comprehensively.

83 **2. Materials and methods**

84 **2.1. Materials**

85 Fresh samples of *N. zanardinii* were collected from the coastal region of Chabahr, in the Sistan and
86 Baluchestan Province, Iran, in February 2017, and were identified by Mr. B. M. Gharanjik (Iranian Fisheries
87 Science Research Institute, Inland Waters Aquatic Stocks Research Center). The seaweeds was carefully
88 washed, oven-dried (40 °C for 72 h), powdered and kept at -20 °C until use. The alcalase, flavourzyme and
89 viscozyme enzymes were from Sigma–Aldrich (USA). Cellulase was purchased from Beijing Solarbio
90 Science & Technology Co., Ltd. (China). The sources of alcalase, flavourzyme, viscozyme and celluclast
91 are *Bacillus licheniformis*, *Aspergillus oryzae*, *Aspergillus aculeatus* and *Trichoderma reesei*, respectively.

92 **2.2. Extraction procedure**

93 **2.2.1. Pre-treatment of *N. zanardinii***

94 In order to remove pigments and small molecules, the milled *N. zanardinii* samples were treated with 85%
95 ethanol (1:10 g/mL) under mechanical stirring overnight at room temperature, with the solvent being
96 changed every 8 h. Following centrifugation (7700 g for 10 min), the supernatant was removed, the seaweed
97 residue was rinsed with acetone and finally dried at room temperature (22±2°C) under a laminar hood for
98 24 h.

99 **2.2.2. Extraction methods**

100 The dried and pre-treated seaweed samples were treated with several non-conventional methods, including
101 alcalase (AL, 2.5 mL from alcalase 2.4 U/g, pH 8, 50 °C, 24 h), flavourzyme (FL, 2.5 mL from flavourzyme
102 500 U/g, pH 7, 50 °C, 24 h), cellulase (CE, 2.5 g from cellulose 3 U/mg, pH 4.5, 50 °C, 24 h), viscozyme
103 (VI, 2.5 mL from viscozyme 100 fungal β-glucanase U/mL, pH 4.5, 50 °C, 24 h), ultrasound (UAE, 200
104 W, 20 kHz, 55 °C, two runs of 20 mins each), microwaves (MAE, 700 W, 90 °C, two runs of 20 mins each),

105 subcritical water (SWE, 1500 W, 150 °C, two runs of 10 mins each), alcalase-ultrasound (EUAE), and
106 simultaneous ultrasound-microwave (UMAE) procedures as well as with conventional methods (hot water
107 extraction, (HWE), 65 °C two runs of 3 h each). The supernatant was removed from the extracted slurry by
108 centrifugation (7700 g for 10 min) after extraction; then it was concentrated by evaporation under reduced
109 pressure at 60 °C. The concentrated extracts were mixed with 1% $CaCl_2$ and the solutions were left 14 h at
110 4 °C in order to precipitate the alginate. After removing the alginate by centrifugation (7700 g for 10 min),
111 the fucoidans were precipitated by ethanol addition to a final concentration of 70%. The crude fucoidans
112 were recovered by centrifugation (7700 g, 10 min), washed with ethanol (99 %, three times) and acetone
113 (twice), and then dried at room temperature under a laminar hood. The fucoidans were weighed and stored
114 at -20 °C until use. The fucoidans obtained were designated as follows: HWE-F (hot water), AL-F
115 (alcalase), CE-F (cellulase), VI-F (viscozyme), FL-F (flavourzyme), UAE-F (ultrasound), MAE-F
116 (microwaves), SWE-F (subcritical water), UMAE-F (ultrasound-microwaves) and EUAE-F (alcalase-
117 ultrasound) [16].

118 **2.3. FT-IR spectroscopy**

119 Fucoidans samples were mixed with KBr and the mixtures were powdered in a porcelain mortar. The
120 powdered samples were loaded into the testing cell and the FT-IR spectra of the samples were recorded in
121 the 400–4000 cm^{-1} region using a Fourier transform IR spectrophotometer for functional group detection
122 (Bruker Instruments, Billerica, USA).

123 **2.4. Chemical composition**

124 The total carbohydrates content was estimated using the phenol–sulfuric acid method at 490 nm, with D-
125 fucose as the standard [17]. The protein content of the different fucoidans was determined using the Lowry
126 method at 720 nm, with bovine serum albumin (BSA) as the standard [18]. The content of total sulfate was
127 analyzed using the $BaCl_2$ gelatin method at 360 nm [19]. Uronic acid content was quantified using the *m*-
128 hydroxybiphenyl method at 525 nm, with D-glucuronic acid as the standard [20].

129 **2.5. Monosaccharide composition**

130 The monosaccharide composition of the extracted fucoidans was determined by gas chromatography-mass
131 spectrometry (GC–MS), according to Tabarsa et al. [21]. Briefly, a polysaccharide sample was hydrolyzed
132 to its constituent monosaccharides with 4 M trifluoroacetic acid (TFA) for 6 h at 100 °C. The hydrolysates
133 were reduced in water using $NaBD_4$, then acetylated with acetic anhydride and finally analyzed using GC–
134 MS (6890N/MSD5973, Agilent Technologies, Santa Clara, CA USA) instrument equipped with a HP-5MS
135 capillary column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, Santa Clara, CA USA).
136 Monosaccharide standards, including fucose, rhamnose, xylose, mannose, galactose, arabinose and glucose,
137 were used according to the instructions.

138 **2.6. Molecular properties**

139 The molecular properties of extracted polysaccharides were determined using a high-performance size-
140 exclusion chromatography column, which was linked to a UV, multi-angle laser-light-scattering and
141 refractive index detection system (HPSEC–UV–MALLS–RI). The column was a SEC column (TSK G5000
142 PW, 7.5mm × 600 mm, Toso-Biosep, Mongomeryville, PA, USA) and was eluted with 0.15 M $NaNO_3$ and
143 0.02% NaN_3 at a flow rate of 0.4 mL/min. BSA was used for the normalization of the MALLS detector and
144 the determination of the volume delay between UV, MALLS and RI detectors. The average molecular
145 weight (M_w), number average molecular weight (M_n) and polydispersity of polysaccharides were calculated
146 from the data collected from MALLS and RI detectors using ASTRA 5.3 software (Wyatt Technology
147 Corp.).

148 **2.7. Antibacterial activity**

149 **2.7.1. Bacterial strain and maintenance**

150 Bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Staphylococcus aureus*)
151 were obtained from the Persian Type Culture Collection (Tehran, Iran). All strains were stored in Tryptic
152 Soy broth (TSB) supplemented with 30% glycerol at -20 °C until use. Before inoculation, all test bacteria
153 were cultured in 10 ml TSB at 37 °C for 24 h. The grown bacteria were separated from the medium by

154 centrifugation (3400 g rpm for 10 min), were washed with 0.85% NaCl solution and centrifuged twice for
155 15 min at 3400 g. After that, the bacterial cell pellet was resuspended in 0.85% NaCl. Subsequently the
156 optical density (OD) of bacterial suspension at 600 nm of a NaCl suspension was adjusted to 0.1 to reach
157 the bacterial suspension of 1×10^8 CFU/mL. The suspension was then diluted to provide a cell concentration
158 of 1×10^5 CFU/ml.

159 **2.7.2. Antibacterial activity of polysaccharides**

160 The antibacterial activity of extracted fucoidans was determined using the agar diffusion method [9]. All
161 bacterial strains were uniformly swabbed on a Tryptic Soy Agar medium surface at a density of 1×10^5
162 CFU/mL. Different concentrations (2, 1, 0.5, 0.25 and 0.125 mg/mL) of sulfated polysaccharides were
163 prepared in distilled water. Twenty microliters of each polysaccharide solution were loaded onto 6 mm
164 diameter sterile paper discs and put onto the surface of the inoculated plates. The plates were incubated at
165 37 °C for 24 h, and the inhibition zone was measured (data expressed as mm). All the experiments were
166 performed in triplicate.

167 **2.7.3. Minimal inhibitory concentration (MIC)**

168 The antibacterial activity of fucoidans was also confirmed by determination of MIC. The active fucoidans
169 (MAE-F, EUAE-F, UMAE-F and SWE-F) against *E. coli* and *P. aeruginosa* were used for determination
170 of MIC. MIC was measured by the broth dilution method as reported by Shanmughapriya et al. [22] with
171 some modifications. Appropriate amount of Tryptic Soy Broth medium containing different concentrations
172 of mentioned fucoidans were transferred into sterile micro tubes and after that the bacterial suspension was
173 added to each tube. Subsequently, the tubes were incubated at 37 °C for 24 h. MIC were recorded as the
174 lowest concentrations of fucoidans at which no visible growth was observed.

175 **2.8. Cell line and virus**

176 African green monkey fibroblastoid kidney cells (Vero) were grown as monolayers in Eagle's minimal
177 essential medium (MEM) (Sigma-Aldrich, Saint Louis, MO, U.S.A.), supplemented with 10% heat
178 inactivated fetal bovine serum (FBS) and 1% antibiotic solution (Penicillin-Streptomycin™, Sigma-

179 Aldrich), at 37°C in a 5% CO_2 atmosphere. The human herpes simplex virus 2 (HSV-2, ATCC® VR-540™)
180 was propagated in Vero cells at 37 °C; once the cytopathic effect had influenced the whole monolayer, the
181 infected cell suspension was collected and the viral supernatant clarified. The virus stock was titrated by
182 plaque assay, as described in Cavalli et al. [23] and stored at −80 °C.

183 **2.9. Cell viability assay**

184 Cell viability was determined using the MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-
185 2-(4-sulfophenyl)-2H-tetrazolium] assay, as previously described [24]. The extracted polysaccharides were
186 re-suspended in water to a final concentration of 25 mg/mL before use. Confluent Vero cell cultures were
187 incubated in 96-well plates with serial dilutions of the investigated molecules in duplicate for 24 hours, 37
188 °C, in a 5% CO_2 atmosphere. Cell viability was determined using the Cell Titer 96 Proliferation Assay Kit
189 (Promega, Madison, WI, USA). Absorbances were measured at 491 nm using a Multiskan™ FC Microplate
190 Photometer (Thermo Scientific™), and cell viability percentages were calculated by comparing the
191 absorbances of treated cells with those of untreated cells. 50% cytotoxic concentrations (CC_{50}) and 95%
192 confidence intervals (C.I.) were determined using PRISM-4 software (GraphPad Software, San Diego, CA,
193 U.S.A.).

194 **2.9. Antiviral activity of polysaccharides**

195 The anti-HSV2 activity of the sulfated polysaccharides isolated from *N. zanardinii* was investigated using
196 a plaque reduction assay on Vero cells [25]. Vero cells were seeded in 24-multiwell plates at 1.0×10^5/well
197 density. The following day, the cells were treated with serial dilutions of fucoidans for 2 hours in MEM 2%
198 FBS. HSV-2 infection was then conducted at a multiplicity of infection (MOI) of 0.001 PFU/cells in the
199 presence of serially diluted extracts for 2 hours at 37°C. Following washing with the MEM medium, the
200 monolayers were overlaid with 1.2%-methylcellulose MEM medium 2% FBS that contained the serial
201 fucoidan dilutions, and were incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. The monolayers were
202 subsequently fixed, stained with 0.1% crystal violet in 20% ethanol, and the HSV-2 plaques were
203 microscopically visualized and counted. Results were reported as percentages of the viral plaques counted

204 in the treated cells in comparison to the control. Acyclovir was tested in parallel as a reference drug for
205 HSV-2. The half-maximal effective concentration (EC_{50}, the concentration of extract that inhibited HSV-
206 2 infectivity by 50%) was calculated by regression analysis using PRISM-4 software (GraphPad Software,
207 San Diego, California, U.S.A.). The selectivity index (SI) was calculated as CC_{50}/EC_{50}. All experiments
208 were conducted in quadruplicate.

209 **2.10. Attachment assay**

210 Prechilled Vero cells were treated with VI-F for 30 min at 4 °C and then infected with HSV-2 at 0.004 MOI
211 for 2 hr at 4 °C in presence of the fucoidan. After three washes with cold MEM to remove unbound virus,
212 cells were overlaid with 1.2% methylcellulose and shifted to 37 °C. After 24-hr incubation, cells were
213 stained, and viral plaques counted [25].

214 **2.11. Statistical analysis**

215 One way ANOVA and Duncan's test ($p< 0.05$) were performed to calculate the differences in extraction
216 yield, and chemical and monosaccharide composition shown by the various extracted polysaccharides.
217 Antiviral data were analyzed using the sum-of-squares F test.

218 **3. Results and discussion**

219 **3.1. Extraction of fucoidan**

220 Table 1 shows the yields of fucoidan extraction from *N. zanardinii*, as generated by the conventional and
221 non-conventional procedures. Extraction type clearly affected fucoidans yield, which varied from 4.28% to
222 13.15%. The fucoidan content of the *N. zanardinii* samples was in the quite wide 0.4-26.3% range, as
223 reported by other authors for the *Arthrothamnus bifidus* (0.4%), *Agarum cribrosum* (1.2%), *Laminaria*
224 *longipes* (2.4%), *Sargassum polycystum* (4.51%), *Sargassum binderi* (6.16%), *Ascophyllum nodosum*
225 (11.3%) and *Undaria pinnatifida* (26.3%) samples. The observed differences in polysaccharide content is
226 likely due to algal species, the algae harvest season, their isolation and even to the analytical method used
227 [26, 27]. As shown in Table 1, SWE exhibited the highest fucoidan yield (13.15%), while the lowest value

228 was obtained using the VI extraction method (4.28%). The results suggested that the non-conventional
229 methods used in the study can be classified into two categories, according to their extraction efficiency; the
230 FL, VI, CE, UAE group displayed low efficiencies, whereas SWE, EUAE, MAE, AL and UMAE exhibited
231 higher extraction power than the HWE reference method. The higher yields provided by AL, as compared
232 to HWE, can be explained by the better cell-wall matrix dismantling displayed by AL over rather prolonged
233 reaction times. These results are similar to those of Zhu et al. [28], who demonstrated that enzyme-assisted
234 extraction furnished higher polysaccharide yields than HWE from *Cornus officinalis*. During MAE
235 extraction, microwaves are absorbed by material components, and the electromagnetic energy is converted
236 into thermal energy. This selective volumetric heating strongly enhances extraction kinetics [29]. Under
237 these conditions, the temperature inside the samples increases, leading to cell rupture in the raw material,
238 which facilitates the diffusion of the intracellular polysaccharides into the solvent [30, 31]. The fact that
239 EUAE provided higher yields than HWE can be explained by alcalase's initial action on the cell wall, before
240 sonication produces high cavitation intensity, streaming and microjets, which lead to faster solvent
241 penetration and matrix solvation [32]. These sequential events can accelerate the release of the intracellular
242 polysaccharides into the solvent and consequently increase the extraction efficiency. Easson et al. [33] and
243 Wu et al. [34] have reported the synergistic effect that is generated between enzymes and ultrasound in the
244 extraction of polysaccharides. The high temperature and pressure used in the SWE method modify the
245 physical properties of water as a solvent. Under these conditions, solvent penetration, capillary effects and
246 cell destruction are all improved, resulting in increased extraction yields [35].

247 **3.2. FT-IR spectroscopy**

248 The FT-IR patterns of the various polysaccharides are shown in Fig 1. Analysis of the FT-IR patterns of
249 the *N. zanardinii* polysaccharides revealed that the main absorption bands were similar in all
250 polysaccharides. These included a strong absorbance peak at 3424 cm^{-1}, from the O-H stretching vibration,
251 a peak at 1420 cm^{-1}, from the asymmetrical bending vibration of CH_3, a peak at 1366 cm^{-1}, from the
252 symmetrical bending vibration of CH_3, a peak at 1250 cm^{-1}, from the sulfate esters (S=O), and a peak at

253 818 cm^{-1}, from the sulfate group (C-O-S). The peaks of the fucoidans extracted in this present study are
254 very similar to the peaks reported for the fucoidans extracted from *Sargassum glaucescens*, *Sargassum*
255 *polycystum* and *Sargassum binderi* in previous works [36, 37, 26].

256 **3.3. Chemical composition**

257 In Table 1 the chemical compositions of the sulfated polysaccharides are reported according to the non-
258 conventional techniques used for extraction. Carbohydrate contents in HWE-F, AL-F, FL-F, CE-F, VI-F,
259 UAE-F, EUAE-F, MAE-F, UMAE-F and SWE-F were 58.13%, 53.55%, 56.19%, 62.04%, 47.5%, 58.65%,
260 52.78%, 51.27%, 45.87% and 54.63%, respectively. SWE-F (4.16%) and AL-F (6.98%) contained the
261 lowest protein amounts. The highest sulfate contents were observed in UMAE-F (27.16%) and AL-F
262 (29.60%). Conversely, SWE-F displayed the lowest sulfate content (11.56%). Uronic acid contents in
263 HWE-F, AL-F, FL-F, CE-F, VI-F, UAE-F, EUAE-F, MAE-F, UMAE-F and SWE-F were 1.2%, 0.42%,
264 0.72%, 0.69%, 0.67%, 1.08%, 0.73%, 0.65%, 1.1% and 1.92%, respectively. The fucoidans isolated from
265 *Sargassum polycystum* by hot water extraction methods contained 38.76% carbohydrates, 22.35% sulphate,
266 3.9% uronic acid and 4.7% proteins [36]. Microwave extracted fucoidans from *Ascophyllum nodosum*
267 contained varying levels of sulfate (6.10-29.33%), which differed according to the extraction time and
268 temperature used [38]. The chemical composition of brown seaweed fucoidans can vary with algal species,
269 population age, environmental conditions, geographic location and seaweed harvest season [36].
270 Furthermore, Dong et al. [39] reported that the chemical composition of extracted polysaccharides depends
271 on the isolation and purification methods used.

272 **3.4. Monosaccharide composition**

273 Compositional analyses of the different fucoidan extracts showed that they were composed of fucose,
274 mannose, galactose, xylose and glucose, whose ratios varied according to the extraction method used. The
275 main monosaccharide found in the polysaccharides extracted from *N. zanardinii* was fucose, similarly to
276 data reported in previously published reports. The monosaccharides observed were present in the following
277 order, from the highest to the lowest content: fucose> mannose> galactose> xylose> glucose (Table 2). The

278 results also showed that rhamnose and arabinose were not found in the fucoidan extracts. This
279 monosaccharide composition was previously reported for fucoidans extracted from *Sargassum polycystum*,
280 *Sargassum angustifolium*, *Sargassum glaucescens* and *Laminaria japonica* species, with ratios that differed
281 according to the extraction method used and algal species [2, 36, 37, 40].

282 **3.5. Molecular properties**

283 The properties of polysaccharides extracted from *N. zanardinii* are shown in Table 3 according to the
284 methods used for extraction. The average M_w of extracted fucoidans were in the 444-1184 kDa range. MAE
285 extracted fucoidans exhibited the highest M_w, while EUAE yielded fucoidans with the lowest M_w. The M_n
286 of HWE-F, AL-F, FL-F, CE-F, VI-F, UAE-F, EUAE-F, MAE-F, UMAE-F and SWE-F were 529.3, 642.2,
287 742.6, 628.65, 777.8, 806.15, 345.7, 643.7, 405.75 and 376.4 kDa, respectively. The polydispersity
288 values for different fucoidans ranged from 1.0 to 1.84. Previous studies have reported a wide range of M_w
289 (64.04-1360 kDa) for fucoidans extracted from *Laminaria japonica*, *Undaria pinnatifida* and *Ecklonia*
290 *maxima* [41, 42]. Algal species, growth conditions and the extraction technique used can all have an effect
291 on the M_w of extracted fucoidans [40].

292 **3.6. Antibacterial activity**

293 The present study also evaluated the antibacterial activity of the various sulfated polysaccharides at
294 concentrations ranging from 0.125 to 2 mg/mL. As shown in Table 3, no sulfated polysaccharide extracted
295 from *N. zanardinii* showed antibacterial activity at concentrations below 2 mg/mL. At 2 mg/mL, however,
296 MAE and SWE polysaccharides inhibited the growth of *E. coli*. Moreover, the polysaccharides isolated by
297 EUAE, UMAE and SWE showed inhibitory effects against *P. aeruginosa* at 2 mg/mL. Chotigeat et al. [43]
298 have previously reported that *Sargassum polycystum* fucoidans displayed inhibitory effects at 6 and 12
299 mg/mL against *E. coli*, *Vibrio harveyi* and *S. aureus*. As shown in Table 4, polysaccharide samples only
300 showed antibacterial activity against Gram-negative bacteria (*E. coli* and *P. aeruginosa*), without any effect
301 on Gram-positive bacteria (*L. monocytogenes* and *S. aureus*). Accordingly, Abdelhedi et al. [44] have
302 previously reported that sulfated polysaccharides isolated from *Mustelus mustelus* displayed higher

303 inhibitory affects against Gram-negative than Gram-positive bacteria. Similarly, Shanmugam et al. [45]
304 have reported that *Sepia brevimana* polysaccharides possessed higher antibacterial activity against Gram-
305 negative bacteria. The antibacterial activity of active fucoidans also was assessed by determination of MIC
306 and the results were shown in Table 4. The MIC for MAE-F and SWE-F against *E. coli* were 1.7 and 1.8
307 mg/mL, respectively. Also, the MIC for EUAE-F, UMAE-F and SWE-F against *P. aeruginosa* were 2, 1.8
308 and 2 mg/mL, respectively. Since there is no report about MIC values of fucoidan, comparison was made
309 between present work and subcritical water hydrolysis from a brown alga *Saccharina japonica* [46]. They
310 reported that the MIC of hydrolysate water with catalyst were ranged from 2.05 to 3.50 mg/mL.
311 Furthermore, the MIC value of de-oiled material with catalyst ranged from 1.60 to 3.20 mg/mL. Previously
312 published reports indicate that the antimicrobial activity of isolated polysaccharides depends on their
313 chemical structure, the sulfate groups and phenolic content [9, 47, 48]. Of note, the extraction method with
314 a broader antimicrobial activity against Gram negative bacteria (SWE-F) is characterized not only by the
315 best yield, but also by the highest Uronic acid % and lowest Protein and Sulfate % in extracted fucoidan
316 composition. Furthermore, the analysis of monosaccharide composition of SWE-F indicate the highest
317 content of fucose and xylose. Further studies are required to investigate at which extent the fucoidan
318 chemical composition affects the antimicrobial activity. Bacterial strain type and bacteria characteristics
319 (cell wall structure and cell composition) also can lead to differences in the microbial activity of compounds
320 [49].

321 **3.7. Antiviral activity**

322 Sulfated polysaccharides are well-known potent anti-viral agents [49]. Their antiviral activity has been
323 reported to be linked to the molecules' anionic features that can inhibit virus adsorption [50]. In order to
324 evaluate the antiviral properties of the extracted sulfated polysaccharides, we screened their inhibitory
325 activity against the reference HSV-2 strain MS using a virus plaque reduction assay on Vero cells. As
326 shown in Table 5, all extracted polysaccharides exhibited potent anti-HSV-2 activity, with EC_{50} values in
327 the 0.027- 0.607 μg/mL range. It is worth noting that the antiviral potencies of AL-F, FL-F, CE-F, VI-F

328 and EUAF-F were similar to that of the conventional extract (HWE-F) (0.031 μg/mL). Interestingly, HWE-
329 F exhibited inhibitory activity against HSV-2 that was higher than that displayed by aqueous polysaccharide
330 extracts from other brown seaweeds [50, 51, 52]. By contrast, a statistically significant lowering of antiviral
331 activity, with respect to HWE-F, was observed in UAE-F, MAE-F, UMAE-F and SWE-F. All the extracted
332 fucoidans, except UMAE-F and SWE-F, were more potent than the positive control, acyclovir. Fucoidan
333 antiviral activity is not a consequence of cytotoxicity as no polysaccharides showed an effect on cell
334 viability at the concentrations used in the antiviral assays. Furthermore, their CC_{50} values were in the 3668-
335 13653 μg/mL range (Table 5). Given their lack of cytotoxicity, all the fucoidans have high selectivity
336 indexes (SI), which range from 13853 to 413727. Our study revealed that VI-F is endowed with the best
337 SI. As reported in Figure 2, VI-F strongly inhibited HSV-2 attachment to Vero cells generating a dose-
338 response curve with EC50 of 0.036 μg/mL, confirming the ability of the extracted fucoidan to inhibit the
339 early phase of HSV-2 infection, as reported in cited references [49,50].

340

341 **4. Conclusion**

342 The yields, molecular properties, antibacterial and antiviral activities of fucoidan extracted from *N.*
343 *zanardinii* by nine different non-conventional and conventional (hot water) methods were evaluated. The
344 highest and lowest fucoidan yields were obtained by SWE and UAE, respectively. Different extraction
345 methods resulted in obtaining fucoidans with various chemical compositions and molecular weights.
346 Fucoidans extracted by MAE and SWE inhibited the growth of *E. coli* and those isolated by EUAE, UMAE
347 and SWE showed inhibitory effects against *P. aeruginosa* at 2 mg/mL. All the extracted fucoidans inhibited
348 HSV-2 infection, and among them VI-F was endowed with the best selectivity index. Further studies are
349 required to investigate at which extent the fucoidan chemical composition affects the antimicrobial and
350 antiviral properties.

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