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Influence of molecular weight fractionation on the antimicrobial and anticancer properties of a fucoidan rich-extract from the macroalgae *Fucus vesiculosus*

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ABSTRACT

The objective of this study was to investigate the antimicrobial and anticancer properties of a fucoidan extract and subsequent fractions isolated from the macroalgae *Fucus vesiculosus*. The fractions obtained (>300 kDa, <300 kDa, <100 kDa, <50 kDa and <10 kDa) could inhibit the growth of *B. subtilis*, *E. coli*, *L. innocua* and *P. fluorescens* when assayed at concentrations between 12,500 and 25,000 ppm. The bacterial growth was monitored by optical density (OD) measurements (600 nm, 24 h) at 30 °C or 37 °C, depending upon on the strain used. The extracted fractions were also tested for cytotoxicity against brain glioblastoma cancer cells using the Alamar Blue assay for 24 h, 48 h and 6 days. The >300 kDa fraction presented the lowest IC_{50} values (0.052% - 24 h; 0.032% - 6 days). The potential bioactivity of fucoidan as an antimicrobial and anticancer agent was demonstrated in this study. Hence, the related mechanisms of action should be explored in a near future.

1. Introduction

Macro algal polysaccharides have recently attracted the attention of the scientific community as a source of hydrocolloids [1] and other water-soluble carbohydrates, i.e., glucans or fucoidans, for next-generation applications in the pharmaceutical and food industries, such as functional food or nutraceuticals amongst others [2]. Nutraceuticals are molecules added to food formulations that are able to display health benefits beyond those of basic nutrition and thus, may play a key role in preventing certain pathological syndromes, avoiding at the same time the secondary effects of the conventional pharmacological treatments [3]. Glucans were studied for their anti-inflammatory properties and their effects on the biochemistry and microbiology of the human gut microbiota [4]. However, amongst all the water-soluble polysaccharides obtained from brown macroalgae, the fucose-containing sulphated polysaccharides or fucoidans have attracted a huge deal of attention for their promising biological properties and possible applications in both nutraceutical and pharmaceutical industries [5]. Fucoidans are described as cell wall polysaccharides produced by brown macroalgae to protect the biomass from environmental stresses [6]. When extracted, these compounds show a wide range of biological activities including antimicrobial [7] and antitumor [8] properties. The exponential growth of food contamination by food-borne and spoilage microorganisms such as *Listeria* spp., *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus subtilis* amongst others has increased the search for natural antimicrobial agents [9,10]. Within all the natural alternatives researched so far, the use of marine polysaccharides like fucoidans extracted from different brown macroalgae

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has shown promising antimicrobial activities against a wide range of pathogens and food microbes [11–13]. Fucoidans have also shown promising anticancer activities when assayed in multiple in vitro and in vivo model systems [14,15], and also synergistic effects with current anticancer therapies. The related anticancer mechanism of action presented by fucoidans includes the induction of cell cycle arrest and apoptosis, together with induction of inflammation through immune system pathways, oxidative stress and stem cell mobilization [14–16]. In general, fucoidans are composed of a backbone of α-(1-3)-linked fucose units or alternating α-(1-3) and α-(1-4) disaccharide units of fucose residues with variable degrees of sulphation [17]. The chemical structure and molecular weight of fucoidans can be associated with biological factors affecting the macro algal biomass [5,17]. The extraction and purification processes used to obtain these polymers can result in mixtures of compounds with variable molecular weights ranging from five to several hundred kDa [17] with variable biological properties and difficulties to maintain the consistency of their production at industrial level [5]. This study aims to explore the antimicrobial and anticancer properties of a fucoidan extract from *F. vesiculosus* supplied by local industry and obtained from production at industrial-scale. The extract was fractionated through multiple molecular weight cut-off (MWCO) membranes and the chemical, antimicrobial and anticancer properties of each fraction were determined aiming to elucidate the most biologically active fucoidan fractions for further applications.

2. Material and methods

2.1. Extract generation

The Fucoidan rich-extract was obtained from Nutramara Ltd. The raw material was Fucus vesiculosus harvested in April 2019 in Co. Galway. The extract was prepared using a proprietary process.

2.2. Molecular weight cut-off fractionation

The fucoidan rich-extract from *F. vesiculosus* was re-suspended in distilled water (5%, *w/v*) and the molecular weight cut-off (MWCO) fractionation of the sample was done using an Amicon® Stirred Cell (Millipore Corporation, MA, USA). The optimal concentration of the samples was achieved by ultra-filtrating the samples through polyethersulfone membranes Biomax™ (Merck Millipore, Darmstadt, Germany) using compressed air (5 bar) and magnetic stirring. The scheme to perform a sequential MWCO using 300 kDa, 100 kDa, 50 kDa and 10 kDa membranes is summarised in Fig. 1. All the fractions were freeze-dried (FD80 model 119, Cuddon Engineering, Blenheim, New Zealand), vacuum sealed and stored at −20 °C for further chemical, antimicrobial and anticancer analyses.

2.3. Chemical analyses

All the chemical analyses were performed in triplicate.

2.3.1. Proximate composition analyses

The extract dry matter was determined by oven-drying the samples (105 °C, 16 h) and the ash content by igniting the samples in a muffle furnace (550 °C, 6 h) following the AOAC.942.05 [18]. The fat content of the extract was determined using an NMR fat analyser (Oracle, CEM Corporation, USA) validated according to the AOAC 2008.06 method [19]. The protein content was determined by measuring the nitrogen content of the samples in a LECO FP 528 instrument (Leco Instruments UKLTD., Cheshire, UK) and applying a conversion factor 4.17 as described for brown macroalgae by Biancarosa et al. in 2017 [20].

2.3.2. Carbohydrate analyses

2.3.2.1. Fucoidan content using high performance liquid chromatography refractive index (HPLC-RI) detector. The fucoidan content of the samples studied was quantified using a HPLC system (Agilent 1200 LC system, Agilent Technologies, Santa Clara, California, USA) fitted with a refractive index detector connected with a guard column (SUPELGUARD H 5 cm × 4.6 mm) and a SUPELCOGEL™ $C_6$10H with 6% cross-linked HPLC carbohydrate column with 30 cm × 7.8 mm (length × I.D.) and 9 μm of particle size (Sigma-Aldrich, Bellefonte, PA, USA). All samples were prepared with a concentration of 2 mg/mL with the running solvent and filtered through 0.45 μm filters (Econo Filter, PTFE, Agilent) and 10 μL were injected on to the column aided by an auto-sampler. The separation was achieved using 0.1% phosphoric acid as the mobile phase at a constant flow rate (0.5 mL/min, 20 min) at 30 °C of column temperature. The identification of fucoidan was performed by comparison of the retention times with those of fucoidan standard from *F. vesiculosus* (≥95% purity, CAS n° 9072-19-9, Sigma-Aldrich, St. Louis, MO, USA) [35]. The integration of the peaks was performed using the software Agilent Chemstation. A standard curve was developed using different concentrations of fucoidan (0.25–2.5 mg/mL). All the analyses were performed in duplicate and the fucoidan content was expressed in g.

2.3.2.2. Total glucan measurement. Total glucans were determined using the kit K-YBGL (Megazyme Ltd., Bray, Ireland). Briefly, 100 mg of yeast β-glucan (positive control) and unknown samples were hydrolysed using a 1 M HCl solution in a water bath (100 °C, 2 h). Samples were neutralised with 2 M KOH, adjusted to 100 mL with sodium acetate buffer (pH 5.0) and centrifuged (1000 g, 10 min). 100 μL of positive control, blanks (sodium acetate buffer, pH 5.0), D-glucose standards (1 mg/mL) and unknown samples were treated using an enzymatic solution containing exo-1,3-β-glucanase (20 units, U/mL) and β-glucosidase (4

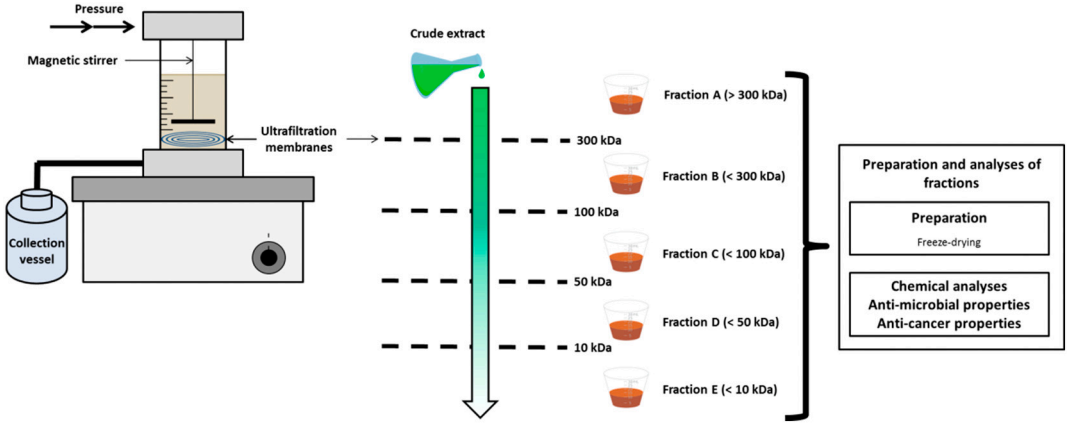

Fig. 1. Scheme summarizing the main equipment and processing steps to perform MWCO fractionation of the fucoidan extract.

U/mL), followed by the addition of glucose-oxidase-peroxidase (GOPOD) reagent. The absorbance of all the samples was read at 510 nm using a spectrophotometer (Epoch, BioTek, Winooski, VT, USA).

2.4. Antimicrobial properties

2.4.1. Microorganisms and culture conditions

The antimicrobial activity of the studied macroalgae fractions was tested using the following strains of bacteria: *Listeria innocua* NCTC 11288, *Escherichia coli* K12 DH5α, *Pseudomonas fluorescens* DSM 50090 and *Bacillus subtilis*. All strains were obtained from ceramic beads in glycerol at −80 °C from the collection of Teagasc Food Research Centre, Ashtown, Dublin, Ireland. Before each assay, a bead of each strain was streaked on Tryptone Soy Agar (TSA, Oxoid, U.K) and incubated for 18 h at either 37 °C in the case of *L. innocua* and *E. coli* or 30 °C for *P. fluorescens* and *B. subtilis*. A single colony was removed from each plate and inoculated into tubes containing Tryptone Soy Broth (TSB, Oxoid, UK) and incubated for 22 h at the appropriate temperature of each bacterial strain (30 or 37 °C). Subsequently, the cells were harvested by centrifugation at 8000 rpm (6 min, 4 °C) and re-suspended in Maximum Recovery Diluent (MRD, Oxoid, UK). The initial concentration of each strain was confirmed by serial dilutions plating on TSA and incubated for 18–20 h at either 30 or 37 °C.

2.4.2. Minimum inhibitory concentration (MIC) assay

The MIC's of the macroalgal fractions were estimated according to the method developed by Smyth et al. [31]. Each fraction was re-suspended in sterile water (10%, w/v) and this initial solution was further diluted in sterile water in order to obtain in the well each of the following concentrations: 5%, 2.5%, 1.25%, 0.625%, 0.3125%, 0.156% and 0.078%. The inoculum of the target microorganism was diluted appropriately in MRD to achieve a concentration in the well of log 6.0 ± 0.5 CFU/mL. 100 μL of each seaweed fraction concentration was mixed with 80 μL of TSB and 20 μL of bacteria in each well of the 96 well flat-bottom microplate. The microplates were covered, incubated during 24 h and then checked for growth using the iodonitrotetrazolium chloride (INT) method as an appropriate indicator of microbial growth [32]. The MIC of each seaweed fraction against the target microorganism was determined as the lowest concentration at which no pink colour appeared. Since some fraction concentrations presented an ambiguous pink colour, the test was complemented with the seeding of a 10 μL loop in TSA to confirm the absence of growth. This assay was repeated twice for each bacterial strain to ensure reproducibility.

2.4.3. Bacterial growth curves and model fitting

The effect of the macroalgal fractions on *L. innocua*, *E. coli*, *P. fluorescens* and *B. subtilis* growth was monitored by optical density (OD) measurements at 600 nm at a regular interval of 30 min using 96 well plates in a temperature-controlled microplate reader spectrophotometer (Epoch 2, BIOTEK, USA), during 24 h at 30 °C or 37 °C depending upon on the strain used. The concentrations used for each macroalgal fraction were based on the MIC's assay results for each microorganism. Each well was inoculated in triplicate with 100 μL of each macroalgal fraction, 80 μL of TSB and 20 μL of bacterial suspension with concentration in the well of log 4.0 ± 0.5 CFU/mL. A control of bacterial growth was performed with TSB and another control of bacterial growth inhibition was performed with gentamicin (initial concentration of 500 mg/L for Gram-negative) and vancomycin (initial concentration of 500 mg/L for Gram-positive). The growth curves were repeated in triplicate for each bacterial strain to ensure reproducibility.

2.5. Anticancer properties

2.5.1. Cell culture

The human brain glioblastoma cancer cell line (U-251 MG (formerly known as U-373 MG) (ECACC 09063001)) cells were obtained from the culture collection of Prof. Michael Carty (Trinity College, Dublin, Ireland). Cells were cultured in Dulbecco's Modified Eagle's Medium-high glucose (Merck, Germany) supplemented with 10% foetal bovine serum (Merck) and 1% penicillin-streptomycin solution (Merck, Germany) in TC flask T75, standard for adherent cells (Sarstedt, Ireland). The cultures were maintained in a humidified incubator containing 5% CO_2 at 37 °C. Culture medium was changed every 2–3 days until 80% confluence was reached. Cells were routinely sub-cultured using 0.25% trypsin-EDTA solution (Merck, Germany). Cells were seeded at a density of 1×10^4 cells/well (24 and 48 h treatment) or 2.5×10^3 cells/well (6 days treatment) (100 μL culture medium per well), in triplicate in 96-well plates (Greiner Bio-One, United Kingdom). Plates were incubated overnight at 37 °C with 5% CO_2 to allow proper adherence. Existing media was removed from each well and cells were then treated with 10% of the 10% (w/v) of each of the different fucoidan fractions and serially diluted from 1 to 0.0078%; 20% dimethyl sulfoxide (DMSO) was used as a positive control and media was used as negative control.

2.5.2. Cell viability assay

The Alamar Blue assay encompasses a fluorometric/colorimetric growth indicator based on the detection of metabolic activity. The system incorporates an oxidation-reduction indicator that both fluoresces and changes colour in response to chemical oxidation of growth medium as a result of cell death. A decrease in cell viability results in a colour change from pink (reduced, fluorescent) to blue (oxidised, non-fluorescent). The Alamar Blue assay (Invitrogen, Ireland) was used to measure the effects of macroalgal fractions extracted against U-251MG cells. After 24 h, 48 h and 6 days of incubation at 37 °C in 5% CO_2, the cells were rinsed once with phosphate buffered saline (Merck, Germany), and then incubated for 2.5 h at 37 °C with 10% Alamar blue and 90% DMEM-high glucose solution. The cell viability was measured by fluorescence (excitation, 530 nm; emission, 590 nm) using a Varioskan™ LUX multimode microplate reader (Thermo Scientific, USA).

2.6. Statistical analyses

For the antimicrobial properties of all the fractions, the primary growth parameters were obtained by plotting and fitting OD600 against incubation time to the modified Gompertz model using the DMFit excel based tool [33]. The maximum specific growth rate (μmax), lag phase (λ, h) were determined using Eq. (1).

$$OD_t = OD_0 + \frac{[OD_{max} - OD_0]}{[1 + e^{(-B(t-M))}]} \quad (1)$$

where, OD_0 and ODmax are the initial and maximum optical density [-]; ODt is the optical density at incubation time (h); B is the maximum relative growth (h^{-1}) at t = M and M is time (h) at which the absolute growth rate was maximum. Maximum relative growth (B, h^{-1}) and M can be used to calculate the SGR and lag phase of the growth curve. The SGR and lag phase for the modified Gompertz model can be calculated by using Eq. (2) [34].

$$\textit{Specific growth rate } (\mu_{max}) = \frac{[OD_{max} - OD_0]}{e} \times B \quad (2)$$

$\textit{Lag phase } (\lambda) = M - \frac{1}{B}$ (Eq. (2)); where "e" is the base for natural logarithms.

Regarding the anticancer properties, all assays were performed in triplicate, independently of each other with a minimum of three replicates per experiment. Data shown is pooled and presented as mean ± SEM (n = total number of replicates) unless stated otherwise. Curve fitting and statistical analysis was performed using Prism 9, GraphPad Software, Inc. (USA). Unless otherwise indicated, significant differences were considered with a *P value <0.05.

3. Results and discussion

3.1. Chemical composition of the fucoidan extract and subsequent fractions

The proximate composition (moisture, ash, crude lipids, protein and total glucans) of the fucoidan extract is summarised in Table 1. In general, the extract revealed a high amount of ash, which can be related with the high content of minerals in macroalgae. The levels of total glucans, lipids and proteins were all at concentrations of <3% in this extract.

The chemical composition of each fraction collected at each molecular weight (>300 kDa, <300 kDa, <100 kDa, <50 kDa and <10 kDa) is summarised in Table 2. Overall, the majority of the water-soluble compounds were retained in the fraction >300 kDa, particularly in the case of fucoidan, with 82.23 ± 3.03% retained in this high molecular fraction. Similarly to other studies evaluating high and low molecular weight fucoidans obtained via MW fractionation and ultrafiltration, the chemical composition and structure elucidation of the most promising fractions will deserve further study and elucidation [21]. In our study, the fractions B to E (Table 2) showed lower fucoidan content, which can be due to the presence of low molecular weight fucoidan passing through the membrane system. In addition, the retention time of these particular samples came after 9 min (Fig. not shown) which can be related with low molecular weight fucoidans. The fucoidan content (g) (Table 2) was determined using HPLC-RI [35], and the resulting chromatograms are presented in Fig. 2b–d respectively. The chromatograms of the standard, whole fraction and fraction A (>300 kDa) showed that the retention time of fucoidan was around 8.7 min respectively. The standard curve was performed using different concentration of fucoidan (0.25–2.5 mg/mL), with a linearity of $R^2 = 0.9964$ (Fig. 2a). It was observed that both the standard and fraction A (>300 kDa) showed one sharp peak, while the chromatogram of the whole fraction sample consisted of an unresolved adjacent peak. This indicates the presence of two components co-eluting together in the crude sample, suggesting that the crude fucoidan isolated from *F. vesiculosus* is highly complex in composition confirming the presence of other components.

In the case of the remaining water-soluble compounds presented in the fucoidan extract from brown macroalgae, the subsequent fractions obtained from the fucoidan extract were also analysed. The accumulation of both protein and total glucans followed in general a similar pattern to the fucoidan content of the extract, although in lower concentrations. Total glucans and proteins were retained mainly in the higher molecular weight fraction. However, the overall concentration of these compounds in the extracts was very low and thus, the levels of total glucans represent 1.61 ± 0.15 g per 100 g of this fraction. The protein content had a similar behaviour to that of total glucans and was present in low concentration in all the fractions generated from this fucoidan extract.

Fucoidan content (g) is expressed in absolute terms as g of compounds per dried weight of fucoidan extract or fractions (A–E). The amount of other minor water-soluble compounds of the extract (protein and total glucans) were also analysed in each fraction. Numbers in brackets indicate the % of compounds retained in each fraction with respect to the original fucoidan extract filtrated.

Table 1
Proximate composition of the fucoidan extract.

Parameters[a]	Fucoidan rich-extract (average ± SEM)
Moisture (%)	4.19 ± 0.09
Ash (%)	17.58 ± 0.07
Crude lipids (%)	2.57 ± 0.12
Protein (%)	2.20 ± 0.01
Total glucans (%)	2.32 ± 0.18

[a] The units of all the parameters are expressed of % or g per 100 g of dried extract.

Table 2
Chemical composition of the fucoidan extract and subsequent molecular weight fractions generated using MWCO techniques.

Fraction	Protein (g)	Total glucans (g)	Fucoidan content (g)
Whole fraction	3.30 ± 0.01 (100%)	2.32 ± 0.18 (100%)	63.26 ± 0.62 (100%)
Fraction A (>300 kDa)	1.72 ± 0.01 (52.05 ± 0.28%)	1.61 ± 0.15 (69.36 ± 5.72%)	50.88 ± 1.87 (80.43 ± 2.96%)
Fraction B (<300 kDa)	1.21 ± 0.03 (36.52 ± 0.78%)	1.00 ± 0.03 (43.15 ± 1.49%)	6.55 ± 0.09 (10.35 ± 0.14%)
Fraction C (<100 kDa)	0.56 ± 0.00 (17.08 ± 0.10%)	0.54 ± 0.03 (23.36 ± 1.38%)	2.85 ± 0.04 (4.50 ± 0.06%)
Fraction D (<50 kDa)	0.30 ± 0.01 (9.06 ± 0.26%)	0.20 ± 0.01 (8.79 ± 0.23%)	1.08 ± 0.08 (1.70 ± 0.13%)
Fraction E (<10 kDa)	0.15 ± 0.00 (4.52 ± 0.03%)	0.12 ± 0.01 (5.36 ± 0.49%)	0.94 ± 0.03 (1.48 ± 0.05%)

3.2. Antimicrobial properties

Fucoidan alongside with laminarin, phlorotannins, alginic acids, etc. have been attracting huge attention amongst the research community due to the multiple bioactivities presented, in particular antimicrobial properties [22]. In our study, the fractions extracted (>300 kDa, <300 kDa, <100 kDa, <50 kDa and <10 kDa) from the fucoidan extract were tested for antimicrobial properties against two Gram-positive (*B. subtilis* and L. *innocua*) and two Gram-negative (*E. coli* and *P. fluorescens*) bacterial strains. Overall, as seen in Fig. 3 all the fractions generated from the fucoidan rich-extract were able to inhibit bacterial growth of both Gram-positive and negative bacteria when assayed at high concentrations (12,500–25,000 ppm), while concentrations of 500 ppm allowed the growth of all bacteria. The growth rate of these microorganisms was drastically reduced with the increase of concentration (Fig. 3) in each and every fraction studied (data not shown). In the case of *B. subtilis* and *P. fluorescens*, the results of the extract at high concentration may be influenced by the effect of other minor soluble compounds present in the extract. At low concentration, the fucoidan content of this extract is the main compound present in these fractions, while the contents of other soluble compounds are minimum. However, at increased concentrations of the extract, the amount of other minor compounds of the extract can considerably increase, thus, influencing bacterial growth at sub-inhibitory concentrations. The increase of biomass observed with *P. fluorescens* can be explained by stress induced through the exposition to the tested substances at sub-inhibitory concentrations, which maybe leads to an extra production of exopolysaccharides (EPS) by the bacterial cell as observed with *P. aeruginosa* exposed to the natural compound, casbane diterpene [23]. Moreover, similarly to the results reported for these fucoidan fractions, variable results have been reported in recent scientific literature examining the antimicrobial properties of fucoidan. Liu et al. in 2017 [12] reported no antibacterial activity of fucoidan extracted from *Laminaria japonica* against *E. coli* and *S. aureus* even at high concentrations (10 mg/mL). The authors reported that the depolymerisation of the molecules following high-pressure methods and thus, the reduction of the molecular weight of the original molecules was key to increase the antibacterial properties of fucoidans through the destruction of cytomembranes and targeting bacterial membrane proteins [12]. Jun et al. in 2018 [11] studied the antimicrobial properties of fucoidan extracted from multiple macroalgae and obtained the highest antibacterial activities against multiple bacteria in those compounds from *F. vesiculosus* with minimum inhibitory concentrations ranging from 125 to 1000 μg/mL. Moreover, the slight differences observed between the fucoidan fractions in this study could also be attributed to the presence of phenolic compounds, such as phlorotannins, and other minor algal components with known antimicrobial properties [24–26]. The growth of bacterial cells in all the fractions generated in this study at

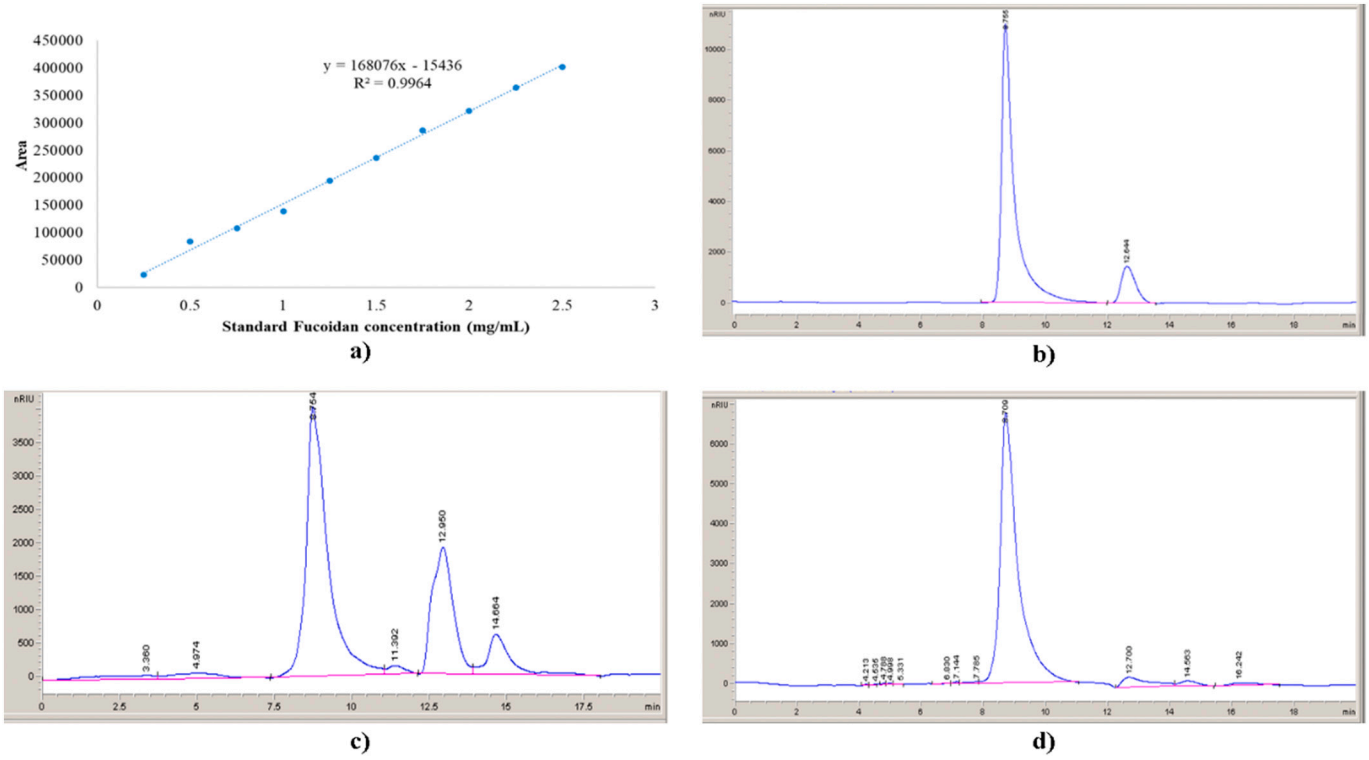

Fig. 2. a) Standard curve of fucoidan; b) HPLC-RI chromatogram of fucoidan standard (2 mg/mL); c) whole fraction and d) fraction A (>300 kDa).

Concentration (ppm)	*B. subtilis*						*E. coli*					
	Fractions						Fractions					
	Fraction A (>300 kDa)	Fraction B (<300 kDa)	Fraction C (<100 kDa)	Fraction D (<50 kDa)	Fraction E (<10 kDa)		Fraction A (>300 kDa)	Fraction B (<300 kDa)	Fraction C (<100 kDa)	Fraction D (<50 kDa)	Fraction E (<10 kDa)	
500												
1560												
3120												
6250												
12500												
25000												

Concentration (ppm)	*L. innocua*						*P. fluorescens*					
	Fractions						Fractions					
	Fraction A (>300 kDa)	Fraction B (<300 kDa)	Fraction C (<100 kDa)	Fraction D (<50 kDa)	Fraction E (<10 kDa)		Fraction A (>300 kDa)	Fraction B (<300 kDa)	Fraction C (<100 kDa)	Fraction D (<50 kDa)	Fraction E (<10 kDa)	
500												
1560												
3120												
6250												
12500												
25000												

Fig. 3. Effect of concentration on microbial (—) growth or (—) inactivation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

500 ppm was monitored over a 24 h period using optical density measurements at 600 nm. Fig. 4 shows the plotted average optical density at each incubation time (30 min) for L. *innocua*, *B. subtilis*, *E. coli* and *P. fluorescens* subjected to incubation with a fixed concentration of the different fucoidan fractions (>300 kDa, <300 kDa, <100 kDa, <50 kDa, <10 kDa) against control (distilled water). The initial OD 600 nm was in a range of 0.001 to 0.038 OD units irrespective of the treatment. As can be seen from Fig. 4, the slope of the exponential phase of treated and control bacterial cells were parallel, indicating that the growth behaviour was relatively similar. Significant increase in lag time was observed only in the case of L. *innocua* when treated with >300 kDa samples compared with the other fractions. The growth parameters obtained by plotting OD 600 nm versus incubation time (h) following the Gompertz model are summarised in Table 3. This model was employed to obtain key growth parameters on the effect of the fucoidan fractions on the behaviour of Gram-positive and negative bacteria including their specific growth rate (μmax, OD unit/h) and lag phase (λ, h). The predicted growth model was tested for the accuracy of model fit

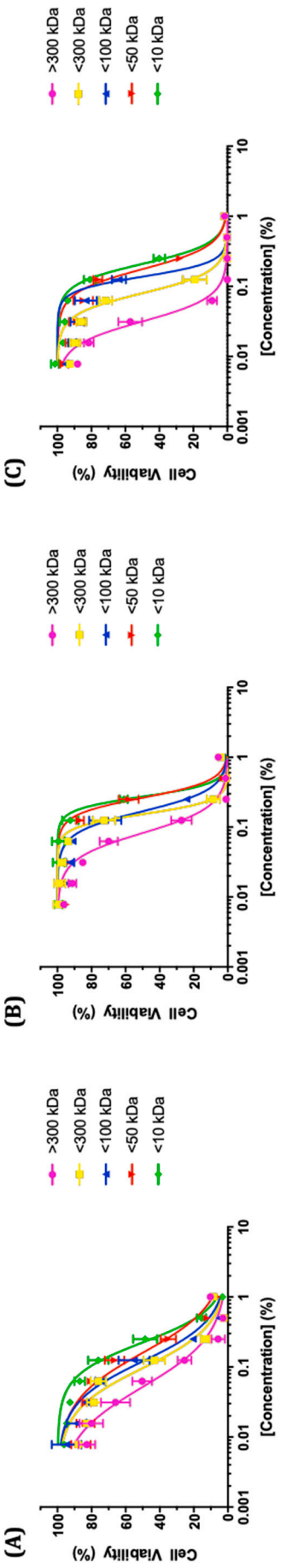

Fig. 4. Growth curves of *B. subtilis* (a), *E. coli* (b), *L. innocua* (c) and *P. fluorescens* (d) over a 24 h incubation period at 30 or 37 °C plotted using Gompertz model fitting subjected to 500 ppm of different fucoidan-rich fractions.

by using R^2 and RMSE values (Table 4). The specific growth rates (μmax, OD units/h) and lag phases (λ, h) obtained from Gompertz model for the different bacteria did not differ between the different fucoidan fractions tested, with exception of L. *innocua*. For that particular microorganism, significant statistical differences ($P < 0.05$) were observed, with the fraction >300 kDa presenting the highest λ value, a fact that can be attributed to a higher susceptibility of this particular microorganism to fucoidans, glucans and other antimicrobial bioactives present at high concentrations in the >300 kDa fraction (Table 2). Despite the lack of statistical differences in the λ amongst most of the microorganisms tested, a relevant result of the present study is the fact that just after approximately 10 h or above (see Fig. 4) the microorganisms started showing some growth at their optimal incubation temperatures (either 30 or 37 °C) amongst all the fractions tested. Many foodborne bacteria form biofilms on the surfaces of food, which can cause food spoilage and disease infections. However, with the supplementation of antimicrobials, as is the case of fucoidans from seaweed, it is expected that some of these issues can be delayed or resolved. The promising results open up new possibilities for research on the use of fucoidan fractions as natural bacteriostatic and bactericidal ingredients for multiple applications, including food preservatives. Studies focusing on this particular use of fucoidan applied to food are scarce in the scientific literature. Poveda-Castillo et al. in 2018 [7] appreciated both a bacteriostatic and bactericidal effect of fucoidan against *Listeria monocytogenes* and *Salmonella typhimurium* in vitro, validating these results by adding these compounds to pasteurized apple beverage to be commercialized under refrigeration.

3.3. Anticancer properties

The anticancer properties of fucoidan have been extensively studied since the 80s. Since then, different studies have shown that these molecules can suppress cancer cells proliferation, induce apoptosis, suppress angiogenesis, etc., both in vitro and in vivo studies [15]. Previous reports have demonstrated that fucoidan molecular weight have been considered one of the main factors that affect in vitro anticancer activities, where low molecular weight has been reported to have higher cytotoxicity due to its ability to increase the solubility and penetration into the cell with reduced toxic effect to normal cells. In our study, the fractions extracted (>300 kDa, <300 kDa, <100 kDa, <50 kDa and <10 kDa) were tested for cytotoxic activities using a glioblastoma multiforme U-251MG cancer cell line with the Alamar Blue assay over an incubation period of 24 h, 48 h and 6 days (see Fig. 5). The IC_{50} values obtained are summarised in Table 5. Overall, the most promising fraction required to inhibit 50% of cell growth is the >300 kDa (Fraction A). This particular fraction presented the lowest IC_{50} value when compared with the other four fractions studied amongst the incubation periods, with values ranging from 0.074% (48 h treatment) to 0.032% (6 days treatment). It can be observed that the IC_{50} of 48 h is higher than the 24 h treatment, which could possibly be because the proliferation of some cells continues in the short-term when incubated with moderately toxic concentrations. That would lead to a perceived increase in viability as these cells divide, but they too can succumb to slower cytotoxicity kinetic. This pattern was also seen with other cytotoxic agents, and we tend to select 6 days as endpoint with the cell line. The Hill slope also agrees with this, as seen in Fig. 5, there is a much steeper Hill slope in the cytotoxicity data for 48 h and 6 days, compared with 24 h, indicating that lower concentrations lead to a population of survivor cells that continues proliferating at this early time point. These results can be attributed to a higher susceptibility of the cancer cells studied to fucoidans, glucans and other anticancer bioactives present at higher concentrations in the >300 kDa fraction (Table 2). Treatments for brain cancer are hindered by the tight junction of the blood brain barrier (BBB), and fucoidans are known to bind to P-selectin which is found on the cell surface of endothelial cells of BBB [27]. P-selectin is also known to be involved in rolling and arresting of leukocytes. Thus, binding of fucoidan to P-selectin can inhibit entry of leukocytes into the brain,

Table 3
Effect of various fractions on microbial specific growth parameter (μ_{max}, h^{-1}) and lag phase (λ, h).

Fractions	*B. subtilis*		*E. coli*		*L. innocua*		*P. fluorescens*	
	μ_{max} (h^{-1})	λ (h)	μ_{max} (h^{-1})	λ (h)	μ_{max} (h^{-1})	λ (h)	μ_{max} (h^{-1})	λ (h)
Fraction A (>300 kDa)	0.042 ± 0.002^{a}	11.424 ± 0.821^{a}	0.062 ± 0.010^{a}	9.044 ± 0.596^{a}	0.071 ± 0.005^{a}	19.97 ± 0.697^{a}	0.153 ± 0.005^{b}	15.56 ± 0.103^{a}
Fraction B (<300 kDa)	0.041 ± 0.013^{a}	9.442 ± 1.1097^{a}	0.056 ± 0.002^{a}	9.581 ± 0.329^{a}	0.127 ± 0.002^{a}	4.49 ± 0.044^{c}	0.19 ± 0.001^{a}	15.81 ± 0.102^{a}
Fraction C (<100 kDa)	0.040 ± 0.001^{a}	9.629 ± 0.369^{a}	0.051 ± 0.008^{a}	10.038 ± 0.142^{a}	0.104 ± 0.010^{a}	13.86 ± 0.740^{b}	0.17 ± 0.009^{ab}	18.73 ± 0.836^{a}
Fraction D (<50 kDa)	0.036 ± 0.009^{a}	8.443 ± 1.844^{a}	0.065 ± 0.008^{a}	9.688 ± 0.114^{a}	0.091 ± 0.005^{a}	14.43 ± 0.462^{b}	0.18 ± 0.001^{b}	17.74 ± 1.508^{a}
Fraction E (<10 kDa)	0.051 ± 0.013^{a}	9.507 ± 0.822^{a}	0.071 ± 0.014^{a}	9.230 ± 0.419^{a}	0.113 ± 0.045^{a}	13.84 ± 0.002^{a}	0.18 ± 0.006^{b}	16.86 ± 0.282^{a}
MSD	0.0436	4.854	0.0376	1.700	0.101	2.442	0.026	3.722

Different letters in the table indicate statistical differences ($P < 0.05$) in the μmax and λ between the different treatments with the different fucoidan fractions.

Table 4
Statistical model parameters for Gompertz model fitted to the growth curves.

Fractions	*B. subtilis*		*E. coli*		*L. innocua*		*P. fluorescens*	
	RMSE	R^2	RMSE	R^2	RMSE	R^2	RMSE	R^2
Fraction A (>300 kDa)	0.008	0.759	0.007	0.996	0.010	0.994	0.025	0.996
Fraction B (<300 kDa)	0.023	0.966	0.003	0.997	0.013	0.958	0.013	0.998
Fraction C (<100 kDa)	0.028	0.948	0.003	0.999	0.012	0.989	0.011	0.995
Fraction D (<50 kDa)	0.011	0.915	0.005	0.999	0.010	0.988	0.018	0.996
Fraction E (<10 kDa)	0.012	0.882	0.006	0.996	0.142	0.994	0.020	0.998

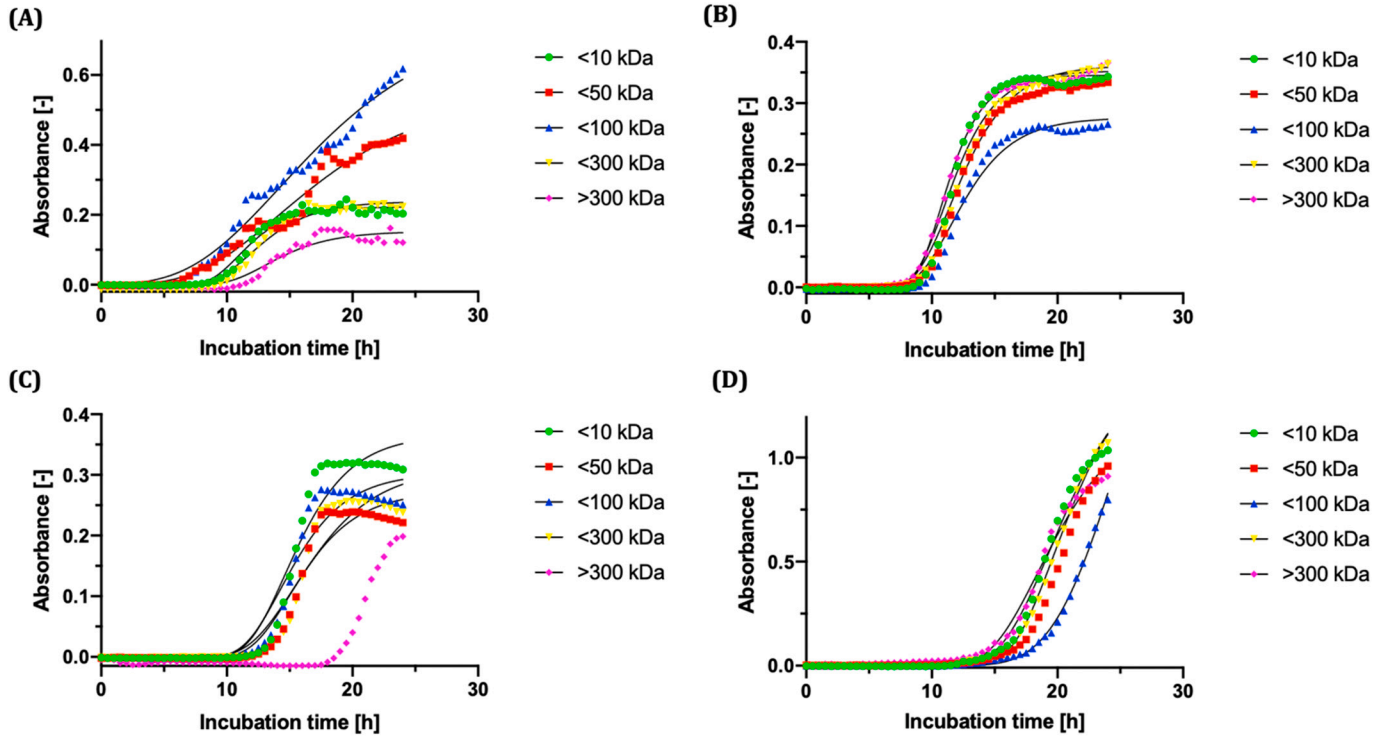

Fig. 5. (A) 24 h (B) 48 h and (C) 6 days treatment of the different fractions in U-251MG cells. Cell viability is plotted against the Log_{10} exponent concentration (% w/v). Data shown was normalised to the untreated control and are shown as mean ± S.E.M. Statistical analysis were carried out using non-linear regression analysis and Two-way ANOVA (*$P < 0.05$) ($n = 3$).

Table 5
Summary of IC_{50} in percentage of fractions extracted %, (*w/v*; g/ml) from fucoidan rich-extract obtained at 24 h, 48 h and 6 days treatment in U-251MG cells.

Fraction	24 h		48 h		6 days		Statistical difference
	IC_{50} (%)	IC_{50} range (%)	IC_{50} (%)	IC_{50} range (%)	IC_{50} (%)	IC_{50} range (%)	
Fraction A (>300 kDa)	0.05203	0.04188 to 0.06423	0.08236	0.07378 to 0.09163	0.03160	0.02838 to 0.03500	$P < 0.05$
Fraction B (<300 kDa)	0.09648	0.07908 to 0.1165	0.1532	0.1432 to 0.1642	0.07983	0.07136 to 0.08887	$P < 0.05$
Fraction C (<100 kDa)	0.1206	0.09963 to 0.1453	0.1686	0.1531 to 0.1856	0.1375	0.1198 to 0.1528	$P < 0.05$
Fraction D (<50 kDa)	0.1599	0.1283 to 0.1973	0.2628	0.2404 to 0.2850	0.1761	0.1546 to 0.1991	$P < 0.05$
Fraction E (<10 kDa)	0.2247	0.1985 to 0.2536	0.2730	0.2601 to 0.2877	0.2093	0.1945 to 0.2249	$P < 0.05$

reducing inflammatory response [28]. Fucoidans have also been proposed to be absorbed by the intestine through SGLT1 and GLUT2 transport system, which can also be a possible mechanism to deliver drugs through BBB [29]. Our study presented promising results in the treatment of GBM with fucoidan rich fractions and could warrant further studies using in vivo models. Yang et al. [30] reported that fucoidan from *Undaria pinnatifida* with a MW of 2200 kDa showed improved anticancer activity (71.3%) compared with the native fucoidans (37.6%), and the authors also found that fucoidan with an MW of 490 kDa increased the anticancer activity (75.9%) in human lung cancer cell line, A549. From the fucoidan results obtained in our study, it is understandable that the fraction >300 kDa presented the highest content of fucoidan and better anticancer results. One the other hand, the lowest fractions with lower fucoidan content (from HPLC-RI results) still present promising results, which can be due to the presence of low molecular weight fucoidan.

4. Conclusions

In the present study, different molecular weight fractions from a fucoidan rich-extract of *F. vesiculosus* were tested for antimicrobial and anticancer properties. The fractions were tested against two Gram-positive (*B. subtilis* and *L. innocua*) and two Gram-negative (*E. coli* and *P. fluorescens*) bacterial strains. In overall, the fractions generated from the fucoidan extract were able to inhibit the bacterial growth of both Gram-positive and negative bacteria when assayed at high concentrations (12,500–25,000 ppm). These promising results suggest new opportunities on the use of fucoidan fractions as natural and green bacteriostatic and bactericidal ingredients to be used by the food industries. Besides, the anticancer investigation using the fucoidan fractions against the growth of glioblastoma multiforme U-251MG cancer cells showed as well very interesting results. The fraction >300 kDa presented the lowest IC_{50} value against the tumoral cells when compared with the other four fractions studied, with values ranging from 0.052% (24 h treatment) to 0.032% (6 days treatment). These results can be attributed to a higher susceptibility of the cancer cells studied to fucoidans, glucans and other anticancer polysaccharides present at higher concentrations in the >300 kDa fraction. These findings need to be corroborated by further investigations as well as by others in vitro and in vivo studies in order to avail fucoidans as future anticancer drugs.

CRediT authorship contribution statement

Eduarda M. Cabral; Conceptualization, Methodology, Formal analysis, Investigation, Writing, **Julie Rose Mae Mondala**; Methodology, Formal Analysis, Investigation; **Márcia Oliveira** Methodology, Resources; **Joanna Przyborska**; Methodology, Formal Analysis, **Stephen Fitzpatrick** Conceptualization, Resources; **Dilip K. Rai** Methodology, Formal Analysis; **Saravana Periaswamy Sivagnanam** Methodology, Formal Analysis; **Marco Garcia-Vaquero** Methodology, Formal Analysis; **Denis O'Shea** Supervision, Writing; **Michael Devereux** Supervision, Writing; **Brijesh K. Tiwari** Conceptualization, Methodology, Validation, Data Curation, Writing, Funding Acquisition, Supervision, Project Administration, **James Curtin** Conceptualization, Methodology, Validation, Data Curation, Writing, Funding Acquisition, Supervision, Project Administration.

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Declaration of competing interest

SF is the Technical Director of Nutramara Ltd., which provided the samples analysed in this study. The authors declare no conflict of interest.

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