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Differential effects of *Fucus vesiculosus* fucoidan on fibroblast and macrophage cell lines inflammatory activation

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ABSTRACT

It is estimated that more than one-third of people aged over 16 reported living with inflammation-related chronic illness. Fucoidan, sulfated polysaccharide, has been proven to modulate immune response and inhibit inflammation in both *in vitro* and *in vivo* research. In the present study the effect of commercially available fucoidan from *Fucus vesiculosus* (with purity 97%) on immune response was investigated. Two cell lines, human fibroblasts (HDFa) and mouse macrophages (J774A.1) were used in the study. Cells were activated with pro-inflammatory stimulus (LPS/IFN-γ or PMA) and treated with fucoidan. Results of NO inhibition assay revealed that fucoidan has anti-inflammatory activity and significantly reduces the level of NO produced by the cells. A significant decrease in secretion of IL-6 from both cell lines was observed. Moreover, fucoidan inhibits IL-12 production by mouse macrophages. The obtained results indicate that fucoidan not only acts as anti-inflammatory compound, but also has immunomodulatory activity. Enhanced secretion of TNF-α and IL-8 from macrophages and fibroblasts, respectively, was also noted. The antioxidant activity of fucoidan has also been studied. Fucoidan demonstrates dose-dependent scavenging properties by DPPH assay. Fucoidan's simultaneous ability to reduce inflammation, activate macrophages, and remove free radicals may be used as a tool to fight chronic inflammation disorders. These all contribute to the effective immunoprevention of cancer or other diseases related to the impaired function of the immune cells.

1. Introduction

Inflammation is the body's innate, physiological response to potentially damaging factors such as stress, bacterial or viral infection or injury. The inflammatory reaction involves cells of the immune system, connective tissue cells, blood proteins (such as C-reactive protein, albumin, transferrin, ferritin, fibrinogen, antithrombin, transcortin) and blood vessels. The purpose of inflammation is to reduce the harmful stimuli, neutralize them and repair damaged tissues. Chronic inflammation ceases to be a physiological process and becomes a pathogenic factor, leading to chronic diseases including among others osteoporosis, cardiovascular diseases, obesity, asthma, bronchitis and cancer [1–5]. The inflammatory reaction mechanism is very complex. Under the influence of external stimuli immunocompetent cells such as macrophages and neutrophils release inflammatory mediators (cytokines and proteins other than cytokines, peptides, glycoproteins, prostaglandins and leukotrienes, nitric oxide, and oxygen free radicals) and signal other cellular components of the immune system initiating a cascade of reactions [6,7]. The most commonly activate inflammatory signalling pathways are NF-κB (nuclear factor-κB), MAPK (mitogen-activated protein kinases), and JAK-STAT (Janus kinases/Signal transducer and activator of transcriptions) [8–10]. Cytokines, secreted proteins with molecular weight less than 40 kDa, play an important role in the immune response and affect growth and cellular proliferation in a wide range of tissues [11–13]. They are produced by a variety of cells in human body, macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and stromal cells. Cytokines include different types of proteins, such as chemokines, interferons, interleukins, tumor necrosis factor (TNF), colony-stimulating factors (CSF). These intercellular signaling proteins are proven to be important as both inflammation-promoting (pro-inflammatory) and inflammation suppressing (anti-inflammatory) mediators [14,15]. Released pro-inflammatory cytokines activate immune cells, which produce and release a range of other cytokines [16]. The term "cytokine release syndrome" or "cytokine storm" regarding inflammatory process upregulation by release of cytokines [17]. The recent results suggest that both inflammation-promoting and inflammation-suppressing cytokines are involved in any immune response [15,18]. Balance of appropriate

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immune activation and suppression, immune homeostasis, is influenced by complex mechanism of the crosstalk between pro-inflammatory and anti-inflammatory interleukins [19]. Interleukins such as IL-1, Il-6, IL-8, Il-11, IL-17, IL-18 TNF-α, TNF-β, IFN-α, IFN-β, IFN-γ belong to the group of pro-inflammatory cytokines. Anti-inflammatory cytokines include IL-10, IL-12, IL-22, IL-37, IL-38, TGF-β.

Seaweeds are considered to be a great source of bioactive compounds (belong to the carbohydrates, proteins, minerals, fatty acids or pigments group) with a wide range of biological activities, such as antibiotic, antioxidant and anti-inflammatory [20–24]. One of them is fucoidan, sulfated polysaccharide found in various marine sources including brown algae or sea cucumbers [25,26]. Fucoidans, regardless of the origin, are mainly composed of fucose backbone and sulphate groups. The other monosaccharides (such as glucose, galactose, mannose, rhamnose, arabinose or xylose), as well as uronic acid, proteins and acetyl groups might be also presented. The ratio of the components and the structure of fucoidan molecule vary depending on the species, season, location and maturity [27–29]. Possible therapeutic potential of fucoidans, as well as their biological properties, have been extensively studied recently. It was reported that these marine-derived polysaccharides have anticancer, antiviral, antimicrobial, anticoagulant, antioxidant, antidiabetic, anti-inflammatory and immunomodulatory activity [30–35]. The above-mentioned biological properties of fucoidan make it considered as a potential pharmaceutical or nutraceutical agent. In this respect, the ability of fucoidan to modulate immune response and inhibit inflammation is particularly interesting. A wide range of both *in vitro* and *in vivo* immunomodulatory and anti-inflammatory properties of fucoidans isolated from different sources was demonstrated in the literature [36–39]. Fucoidan has been demonstrated to reduce oxidative stress, regulate expression of transcription factors, pro-inflammatory enzymes and matrix metalloproteinases and influence complement system [40]. All these biological activities of the sulphated polysaccharide contribute to its immunomodulatory and anti-inflammatory properties. Two classical inflammatory signalling pathways, NF-κB and MAPK, and the inhibition of pro-inflammatory cytokines seem to be possibly involved in the mode of action of fucoidan in the contest of its anti-inflammatory activity [41–43]. It was demonstrated that fucoidan from different marine sources reduce level of inflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6) and other inflammation mediators (nitric oxide, prostaglandin E2) in various cellular models, among others, mouse macrophages RAW264.7, BV2 microglia cells, human keratinocytes, human intestinal Caco-2 cells, human mononuclear U937 cells [36,38,39,41,42,44–46]. The ability of fucoidan to modulate immune response was also observed *in vivo* [47–49].

The potential of fucoidan isolated from brown seaweed *Fucus vesiculosus* to reduce inflammation was analyzed in the present study. The effect of tested polysaccharide on the level of inflammation-related markers, such as nitric oxide, interleukins 6, 8 and 12 and tumor necrosis factor alpha was investigated in the activated human dermal fibroblasts (HDFa) and mouse macrophages (J774A.1). To the best of our knowledge, the immunomodulatory activity of *Fucus vesiculosus* fucoidan was never before analyzed using a dermal fibroblast cellular model. As skin is in direct contact with the external environment and is susceptible to damage when exposed to stimuli, it plays a key role in immunity [50,51]. Dermal layer cells, fibroblasts, actively participate in cellular immune response in connective tissues, when activated by signals occuring at inflammatory sites (such as cytokines and products of bacteria metabolism)[52]. The correlation between composition and structure of fucoidan and its biological activity has been reported in the literature [27,53,54]. Seaweed species, season and place of harvesting, chemical characteristics as well as extraction technique can significantly influence obtained biological effect. In this study commercially available fucoidan extracted from *Fucus vesiculosus* was used to assess its effect on immune response. The purity of tested fucoidan according to the CoA was 97 %. Proximate compositional analysis, elemental analysis, FTIR spectroscopy and thermogravimetric analysis/differential scanning calorimetry were used for fucoidan chemical characterization. Moreover, molecular mass was determined using size exclusion chromatography.

2. Materials and methods

2.1. Materials

Fucoidan from *Fucus vesiculosus* (F8190, Lot # SLBZ7467; ≥95 %) was purchased from Sigma-Aldrich Co. Ltd. (Saint Louis, MO, USA). According to the Certificate of Analysis, purity of tested fucoidan was 97 %.

2.2. Proximate compositional analysis

Total protein content was determined using the commercially available assay kit (Bio-Rad, California, USA) following the manufacturer's recommendations. This colorimetric test is based on the Bradford dye-binding method[55]. Polyphenol content was estimated by Folin-Ciocalteau assay. The method was adapted from Zhang at al. [56]. Total sugar content was measured by the method developed by DuBois and al. [57]. Total glucans were measured using the Mushroom and Yeast Beta-Glucan Assay (K-YBGL; Megazyme Ltd., Bray, Ireland) according to the manufacturer's protocol. The kit was optimised for hydrolysis of sample with sulfuric acid as described by McCleary and Draga [58]. Uronic acid content was measured based on the method described by Bitter and Muir [59]. Mannitol concentration was determined using D-Mannitol Assay Kit (K-MANOL; Megazyme Ltd., Bray, Ireland) following the manufacturer's recommendations.

2.3. Elemental analysis

Elementary analysis of total carbon, hydrogen, nitrogen and sulphur in tested fucoidan was carried out in the Institute of Organic Chemistry, The Polish Academy of Sciences (Kasprzaka 44/52, 01–224 Warsaw, Poland) using elemental analyser (Vario EL III). Measurements were based on catalytic combustion of samples at 1150 °C in an atmosphere of oxygen. After removing interfering gases (such as volatile chlorides), the combustion gases were separated in an adsorption column and continuously measured with a thermal conductivity detector (TCD).

2.4. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra of the tested *Fucus vesiculosus* fucoidan were analysed using FT-IR spectrophotometer equipped with an ATR (attenuated total reflectance) sampling device containing diamond crystal. Analysis was performed using Shimadzu IRTracer-100.

The spectra were recorded at an absorbance mode of 550 to 4000 cm^{-1}. Analysis was performed in the Institute of Organic Chemistry, Polish Academy of Sciences, Kasprzaka 44/52, 01–224 Warsaw, Poland.

2.5. Thermogravimetric analysis/differential scanning calorimetry

Thermogravimetric analysis/differential scanning calorimetry (TGA/DSC) of the fucoidan was carried out in Laboratory for Soft Matter Research at Institute of Physical Chemistry, Polish Academy of Sciences, Kasprzaka 44/54, 01–224 Warsaw, Poland. The range of temperature for the sample was 25–600 °C, heating rate was 3 °C/minute with protective gas flow- nitrogen.

2.6. Molecular weight analysis by size exclusion chromatography (SEC)

To determine the molecular weight of the tested *Fucus vesiculosus* fucoidan, two steps were required. First, the dextran standards and the fucoidan were fractionated on a size exclusion column, and then all fractions were subjected to analysis for total sugar content by

phenol–sulphuric acid assay [57]. The fractionation was performed using a Superdex 200 Increase column (10 x 300 mm, GE Healthcare) and a ÄKTA purifier FPLC system (Sweden) fitted with a pump (P-900), detector (OV-900) and fractionator (F-950). The running eluent contained 0.2 M sodium acetate and 0.15 M sodium chloride, adjusted to pH 4.5. The flow parameter was 0.4 ml/min. The fucoidan concentration was 20 mg/ml. The injection volume was 0.1 ml at room temperature. Dextrans from *Leuconostoc mesenteroides* (Fluka) with molecular weights of 12, 25 and 80 kDa and dextran blue 2000 kDa from *Leuconostoc* ssp. (Fluka) calibrated the column. Fractions of 0.5 ml were collected, and aliquots were analysed for total sugar content. The original phenol–sulphuric acid assay used was according to Dubois et al. [57]. Partition coefficient values (K_{av}) for all dextran standards were calculated using the formula below:

$$Kav = \frac{V_R\text{-}V_O}{V_C\text{-}V_O}$$

V_o = void volume; V_c = bed volume; V_R = retention (elution) volume of separated sample

2.7. DPPH scavenging activity

The scavenging activity of DPPH free radicals was assessed according to Mensor et al. [60]. 2.5 ml of fucoidan samples (100–4000 μg/ml) were combined with 1 ml of 0.3 mM methanolic DPPH solution. Samples were mixed vigorously and left for 30 min at room temperature, and the absorbance was measured at 518 nm. Ascorbic acid was used as a positive control. The % antioxidant activity was calculated using the following equation:

$$\%\ \text{antioxidant activity} = \ 100\text{-}[\text{antioxidantactivity} = 100 - [\frac{\text{Absorbance of sample - absorbance of blank}}{\text{Absorbance of control}} \text{x } 100]$$

2.8. Cell culture

The human dermal fibroblasts cell line, HDFa, was purchased from Gibco® Cell Culture. Cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM; GibcoTM, Thermo Fisher Scientific, Waltham, MA, USA) containing 5 % fetal bovine serum (FBS; GibcoTM, Thermo Fisher Scientific, Waltham, MA, USA) and 2 mM L-glutamine (Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in a 5 % CO_2 humidified atmosphere. The immortalized murine macrophages, J774A.1, were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in complete ATCC-formulated Dulbecco's Modified Eagle's Medium with 10 % FBS (Gibco®, Thermo Fisher Scientific, Waltham, MA, USA).

2.9. Cell viability assay

Murine macrophages J774A.1 and human dermal fibroblasts HDFa were seeded at a density of $5x10^4$ cells per well in 96-well plates in 100 μl of complete media. Cells were allowed to adhere overnight. Treatment media was prepared to contain an appropriate concentration of tested fucoidan from *Fucus vesiculosus*. Treatments were performed in triplicate. Cells were cultured for a further 24 h. Media only was used to treat control cells. After exposure for the desired period of time, 11 μl PrestoBlue®Cell Viability Reagent (Thermo Fisher Scientific, Walthman, MA, USA) was added to each well of the 96-well plate. Plates were incubated in the dark for 2 h at 37 °C. Fluorescence was read using a 560 nm excitation/590 nm emission filter set (10 nm bandwidth) with a Thermo Scientific™ Varioskan LUX spectrophotometer. Fluorescence data in wells containing cells were corrected for background fluorescence using cell-free media control replicates.

2.10. Cell treatment

J774A.1 and HDFa were cultured in a 96-well plate ($4x10^4$ cells/well; 200 μl) for 24 h. Cells were subjected to 2 h stimulation with the tested fucoidan at concentrations 0.25; 0.125 and 0.0625 mg/ml. Murine macrophages were stimulated with 100 U/ml interferon gamma (IFN-γ; BD Biosciences Franklin Lakes, NJ, USA) and 20 ng/ml lipopolysaccharide *E. coli* O111:B4 (LPS; Sigma-Aldrich Co. Ltd., Saint Louis, MO, USA) and human dermal fibroblasts with 10 mM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich Co. Ltd., Saint Louis, MO, USA) for further 24 h. Supernatants were used to assess production of inflammation markers. Two well-known anti-inflammatory agents, docosahexaenoic acid (DHA; Sigma-Aldrich Co. Ltd., Saint Louis, MO, USA) and dexamethasone (Dex; Sigma-Aldrich Co. Ltd., Saint Louis, MO, USA) were used as positive controls.

2.11. Cellular antioxidant activity (CAA) assay

HDFa were seeded in black, clear bottom 96-well plates ($2.5x10^4$ cells per well) in 100 μl of complete cell culture media and allowed to adhere overnight. Cell culture media was carefully removed, and the cells were washed twice with PBS. Cells were treated with the tested samples (sterile-filtered) in medium containing 50 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA; Sigma Aldrich). After 60 min of incubation, 0.25 mM hydrogen peroxide was added to the wells for 60 min. Supernatants were removed from the wells, the cells were washed with PBS. Fluorescence was read using a 485 nm excitation/535 nm emission filter set with a Thermo Scientific™ Varioskan™ LUX spectrophotometer.

2.12. Nitric oxide production (Griess assay)

50 μl of the cell culture supernatants were transferred into a 96-well plate, combined with an equal volume of Griess reagent (Sigma-Aldrich Co. Ltd., Saint Louis, MO, USA) and incubated for 10 min at room temperature. The optical density was measured at 550 nm using a Thermo Scientific™ Varioskan LUX spectrophotometer. $NO^{\cdot}$ concentration was evaluated from a comparison to sodium nitrite standard curve freshly prepared in culture medium over the working range of the assay, 3.125 – 200 μM.

2.13. Enzyme-Linked immunosorbent assay (ELISA)

J774A.1 cell culture supernatants were assayed for TNF-α, IL-12 and IL-6. HDFa cell culture supernatants were assayed for IL-6 and IL-8. Cytokines measurement was performed using ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.14. Statistical analysis

All results were expressed as the means ± SEM from three independent experiments (three biological and technical replicates). GraphPad Prism software version 9 (GraphPad Software, CA, USA). One-way ANOVA followed by Dunnett's multiple comparison test was used

Table 1
Proximate composition of the fucoidan from *Fucus vesiculosus*.

Parameters[a]	*Fucus vesiculosus* fucoidan (average ± SD)
Total sugars (%)	86.76 ± 0.96
Polyphenols (%)	0.10 ± 0.03
Protein (%)	n.d.
Total glucans (%)	1.33 ± 0.56
Uronics (%)	5.32 ± 0.96
Mannitol (%)	0.43 ± 0.06

[a] Parameters are expressed of % or g per 100 g of fresh extract.

to analyze significant differences ($p < 0.05$) between the mean values of the individual group.

3. Results

3.1. The proximate composition of fucoidan from Fucus vesiculosus

The proximate composition (sugars, protein, polyphenols, total glucans, uronics and mannitol) of the *Fucus vesiculosus* fucoidan is summarised in Table 1.

Carbohydrates were the main fraction of the fucoidan extract and constitutes 86.76 ± 0.96 % of total organic matter. The second and third most abundant components of the extract were uronics (5.32 ± 0.96 %) and total glucans (1.33 ± 0.56 %). The mannitol and polyphenols content were established as 0.43 ± 0.06 % and 0.10 ± 0.03 %, respectively. Proteins were not detected.

3.2. Elemental analysis of fucoidan from Fucus vesiculosus

As can be seen in Table 2, tested *Fucus vesiculosus* fucoidan contained no nitrogen, which explains why the presence of proteins was not confirmed in the tested fucoidan (Table 1).

The sulphur content was estimated as 7.68 ± 0.12 %. In order to calculate the quantity of sulphate ester, multiplication factor of 2.99582 was used [61], therefore sulphate ester content in fucoidan extract was established as 23.01 ± 0.36 %.

3.3. Fourier transform infrared spectroscopy (FT-IR)

Normalised FT-IR spectra (550–4000 cm^{-1}) of the *Fucus vesiculosus* fucoidan are presented in Fig. 1.

The major peak at 3443 cm^{-1} was assigned for stretching OH group of monosaccharide monomer [62], whereas the peak at 2938 cm^{-1} was associated with stretching and bending vibrations of the alkyl groups (–CH_2–, CH_3) [63]. These two peaks were the most frequent spectral features indicating OH and CH stretching of polysaccharides [64].

Spectra characteristic for the uronic acid group were also identified. The peak at 1639 cm^{-1} confirmed the presence of a C = O stretching band of the carbonyl group. According to Sinurat et al. (2016), this absorption area indicated the presence of uronic acids [65] A minor peak in the region of 1500 cm^{-1} was also interpreted as symmetric stretching vibration of C-O within –COOH of uronic acids [66]. The presence of uronic acids in the tested fucoidan was confirmed by the chemical analysis (Table 1).

Table 2
Elemental analysis (CHNS) of tested fucoidan.

Elements[a]	*Fucus vesiculosus* fucoidan (average ± SD)
Carbon (%)	23.60 ± 0.01
Hydrogen (%)	4.81 ± 0.02
Nitrogen (%)	n.d.
Sulphur (%)	7.68 ± 0.12

[a] Elements are expressed of % or g per 100 g of fresh extract.

The presence of sulphate groups in the tested sample was also identified. The large peak at 1255 cm^{-1} was interpreted as sulphate ester groups (S = O) [67,68], whereas the peaks at 1167 and 1132 cm^{-1} indicated the presence of S = O stretching of alkyl sulfoxide [69]. Another peak characteristic for sulphated polysaccharide emerged at 835 cm^{-1} and was identified as a bending vibration of the C-O-S group as a complex substitution of 4-sulfate and 6-sulfate monosaccharide units [63].

Peaks at 1015 and 962 cm^{-1} were determined as stretching vibrations of the glycosidic links (C-O-C) [62,70]. A characteristic band for deoxy sugar, such as fucose, was identified at 692 cm^{-1} [61].

3.4. Thermogravimetric analysis/differential scanning calorimetry (TGA/DSC)

The TGA/DSC was used to determine the thermal stability of the tested fucoidan. The weight loss of the sample following continuous heating to 600 °C is depicted in the graphs (Fig. 2).

At 33.52 °C, thermal breakdown started and conclusions were reached at 500 °C. The TGA curves demonstrated that fucoidan breaks down into three phases. The first process involved removing the water that has been physically absorbed, while the second step involved removing the water that has been chemisorbed simultaneously with the beginning of the fucoidan molecule disintegration. The breakdown of polysaccharide macromolecules continued in the third stage [71]. The first phase of decomposition occurred between 33 °C and 226 °C and corresponded to 11.08 % moisture loss. This effect is shown as a single endothermic transition at a peak temperature (Tpeak) of 45.81 °C, as seen in the DSC thermogram. Further thermal degradation of fucoidan occurred in two overlapping steps and involved several strong exothermic processes, as can be observed from the DSC curve (Fig. 2). The second and third steps of thermal decomposition represented minor and major evaporation processes, respectively. The second step started at 226 °C and ended at 231 °C with a weight loss of 21.49 %, while the weight loss at the end of the third step at 280 °C was 9 %. The DSC pattern suggested that the structure of the fucoidan was amorphous in nature [72].

3.5. Molecular weight analysis by size exclusion chromatography (SEC)

The eluting profile from the size exclusion chromatography of the tested fucoidan is shown in Fig. 3.

The calibration curve of the dextran standards yielded a correlation factor of $R^2 = 1$, with a linear formula of y = -0.217x + 2.5864. This equation was used to determine the molecular weight of the fucoidan fractions. The average molecular weight of the fucoidan was estimated to be 42 kDa by gel filtration with the Superdex Increase 200 column against standard dextran.

3.6. DPPH scavenging activity

The antioxidant potential of *Fucus vesiculosus* fucoidan was investigated by the DPPH scavenging assay. DPPH, a free radical stable at room temperature, is reduced by antioxidant compounds. The results are presented in Fig. 4.

All tested concentrations of fucoidan (0.1–4 mg/ml) exhibited the ability to scavenge DPPH (IC50 = 1.86 mg/ml). The DPPH scavenging activity of tested sample was lower in comparison to the positive control, ascorbic acid. The percentage antioxidant activity of both tested samples, fucoidan and ascorbic acid, increased in a dose-dependent manner.

3.7. Cell viability assay

The results of the viability assay performed on murine macrophages and human dermal fibroblasts are presented in Fig. 5A and 5B, respectively.

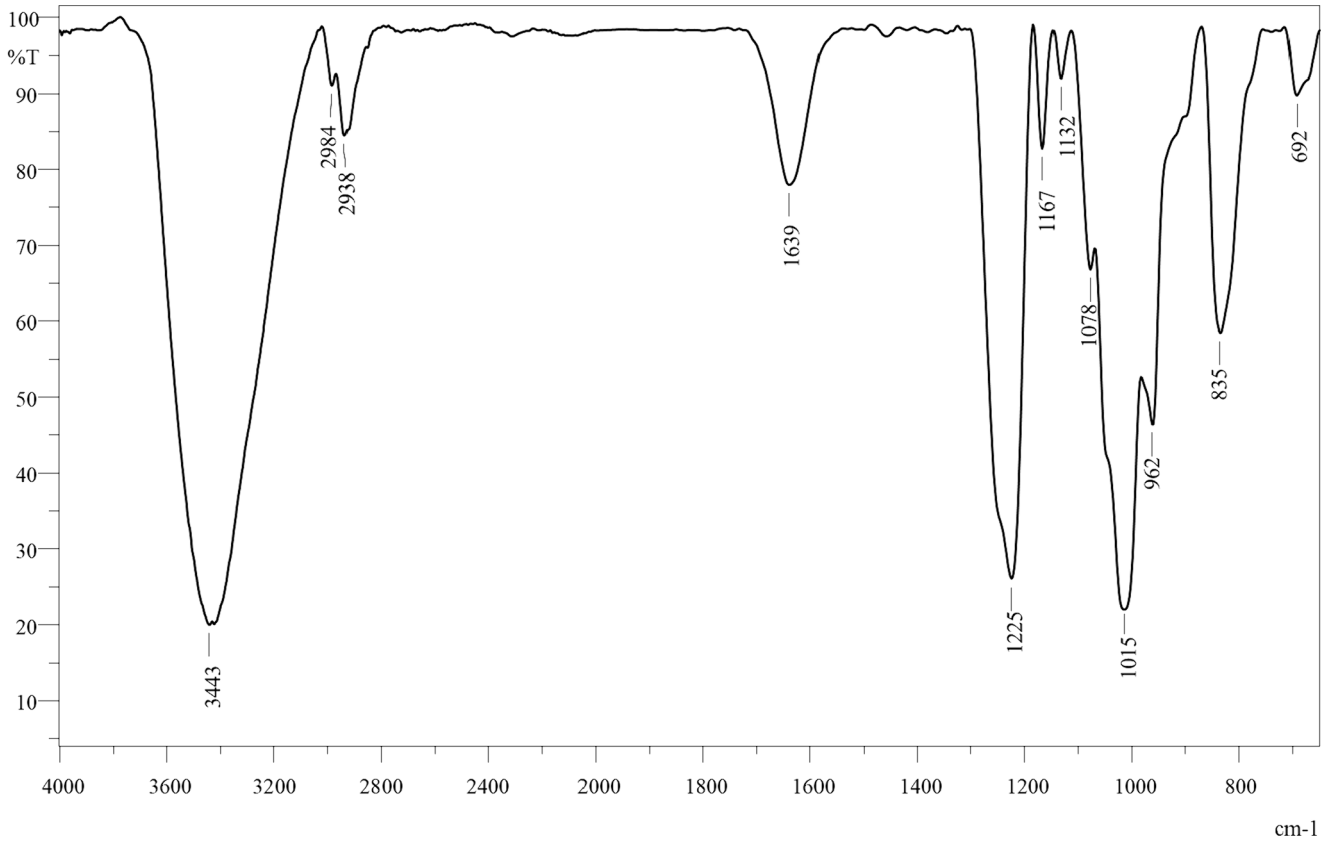

Fig. 1. Fourier transform infrared (FT-IR) spectra of the *Fucus vesiculosus* fucoidan. The IR spectra show a plot of wavenumbers (cm^{-1}) vs. percent of transmittance (%T).

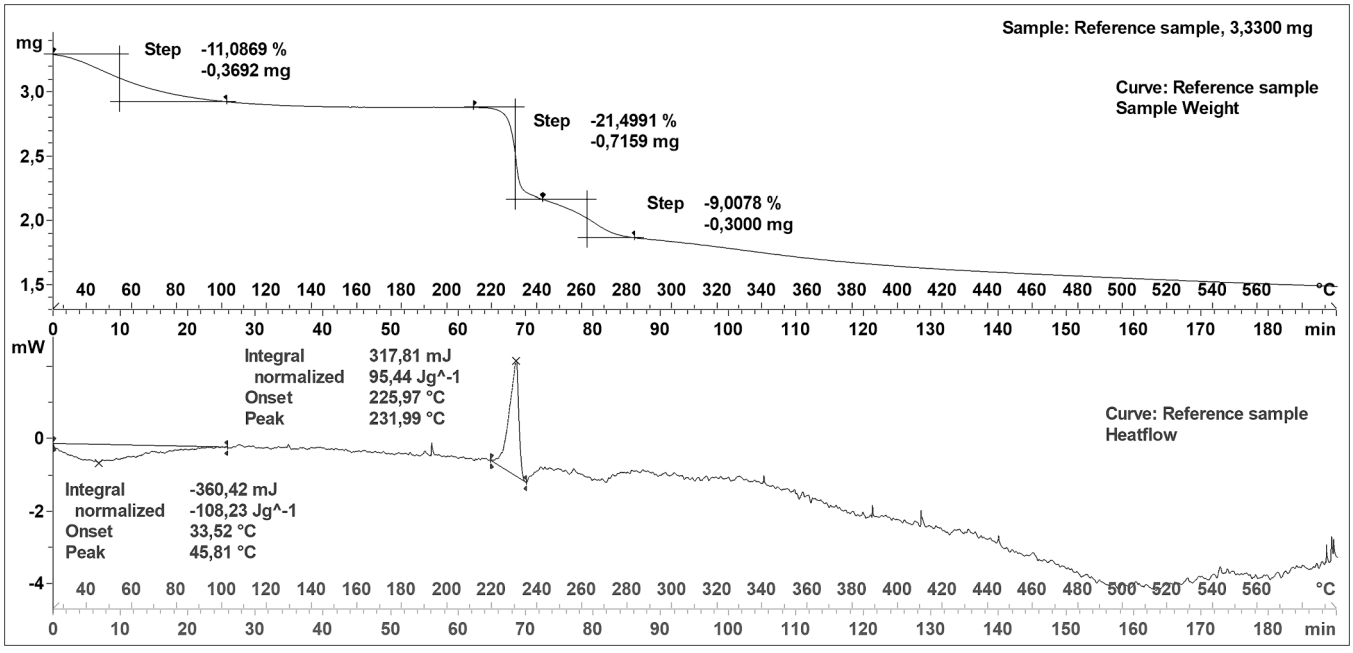

Fig. 2. Thermogravimetric analysis and differential scanning calorimetry (TGA/DSC) of the *Fucus vesiculosus* fucoidan. The TGA graph (upper) represents the mass (mg) as a function of time (min) at a given temperature (°C). The DSC graph (lower) represents the heat flow (mW) as a function of time (min) at a given temperature (°C).

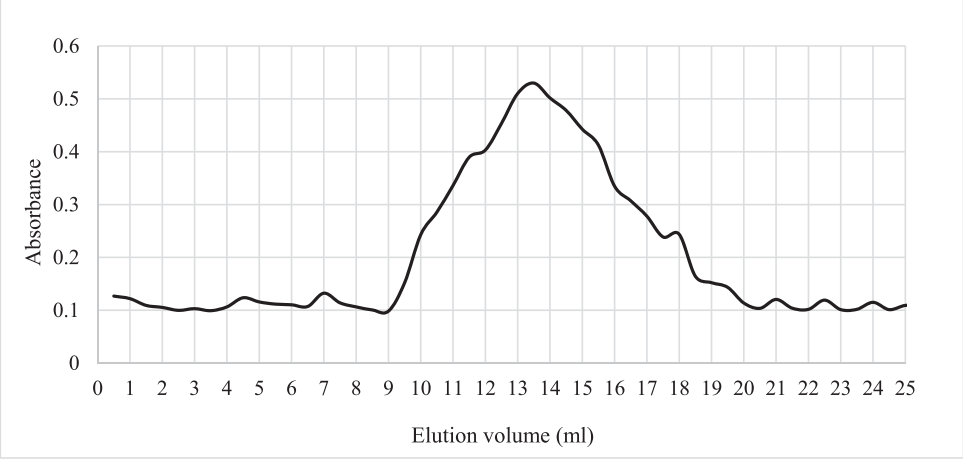

Fig. 3. A size-exclusion chromatogram of the *Fucus vesiculosus* fucoidan on a Superdex 200 Increase column. The graph represents the absorbance of the fractions (ml) at 490 nm.

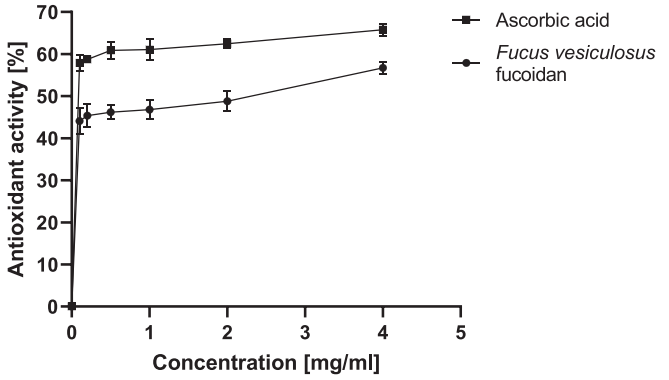

Fig. 4. Scavenging effects on DPPH radical by *Fucus vesiculosus* fucoidan and ascorbic acid, used as a positive control. Values correspond to mean ± SEM of three independent experiments.

Fucus vesiculosus-derived fucoidan significantly decreased viability of both tested cell lines when used at the three highest concentrations, 2, 1 and 0.5 mg/ml. None of the other working concentrations of the tested compound showed cytotoxic effects against human dermal fibroblasts and murine macrophages. Moreover, fucoidan used at the concentrations of 0.25 mg/ml and 0.125 mg/ml significantly increased viability of dermal fibroblasts (124 % and 113 %, respectively; Fig. 5A). In the case of murine macrophages, three concentrations of tested sample, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml, caused statistically significant increase in cell viability (138 %, 134 % and 120 %, respectively; Fig. 5B). Based on the obtained results, three concentrations of *Fucus vesiculosus* fucoidan were chosen for the further experiments, 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml.

3.8. Cellular antioxidant activity (CAA) assay

The ability of *Fucus vesiculosus* fucoidan to protect the dermal cells from oxidative stress by reducing the level of reactive oxygen species was measured using DCF assay. The results of the assay, as presented in Fig. 6, indicate that tested fucoidan exhibits antioxidant properties.

Bars correspond to mean ± SEM of three independent experiments. **** $p < 0.0001$ vs. hydrogen peroxide-treated cells.

Tested sample significantly reduces the level of intracellular reactive oxygen species at all three working concentrations. The obtained level of ROS, determined by DCF fluorescence intensity, was 51 %, 52 % and 46 % for fucoidan 0.25 mg/ml, 0.125 mg/ml and 0.0625 mg/ml,

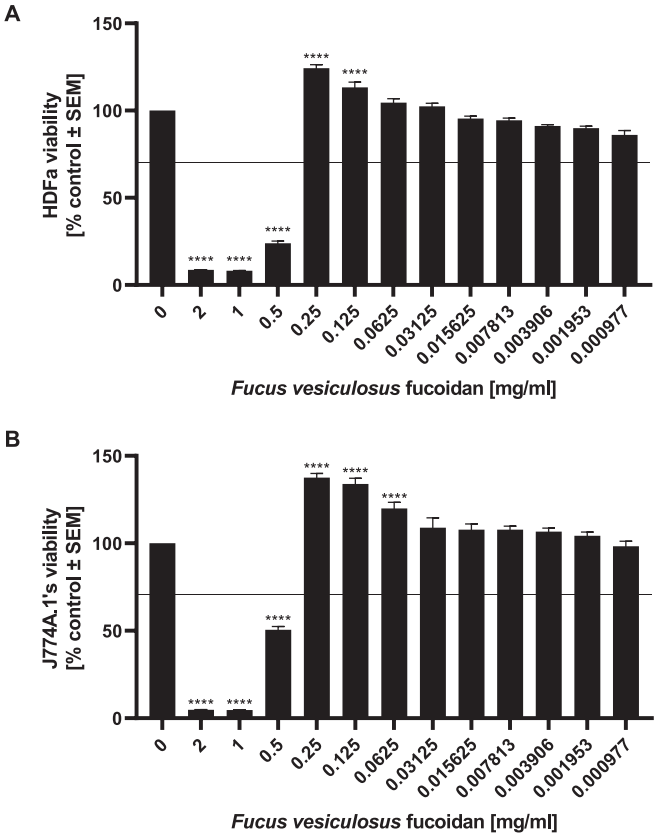

Fig. 5. Effect of the tested fucoidan from *Fucus vesiculosus* on the human dermal fibroblasts (HDFa; A) and murine macrophages (J774A1; B) viability after 24 h incubation. The data are presented as the mean ± SEM from three independent experiments. Statistical significance in comparison to the negative control (untreated cells) was assessed using one-way ANOVA followed by Dunnett's multiple comparison test; ****$p < 0.0001$. The black line indicates the cytotoxicity limit according to ISO 10993–5.

respectively. *Fucus vesiculosus* fucoidan exhibits significantly weaker antioxidant activity then positive control, 100 μg/ml ascorbic acid ($p = 0.006$ for 0.0625 mg/ml fucoidan and $p < 0.0001$ for the remaining two concentrations).

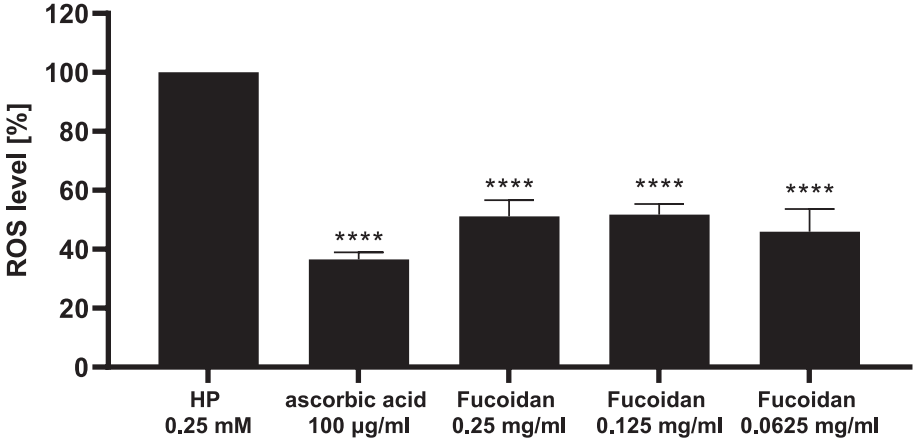

Fig. 6. Intracellular antioxidant activity of *Fucus vesiculosus*-derived fucoidan measured by DCF assay. HDFa were treated with different concentrations of tested samples for 1 h. Ascorbic acid at a concentration of 100 µg/ml was used as a positive control. Results are presented as a percentage of the hydrogen peroxide-treated cells (HP 0.25 mM).

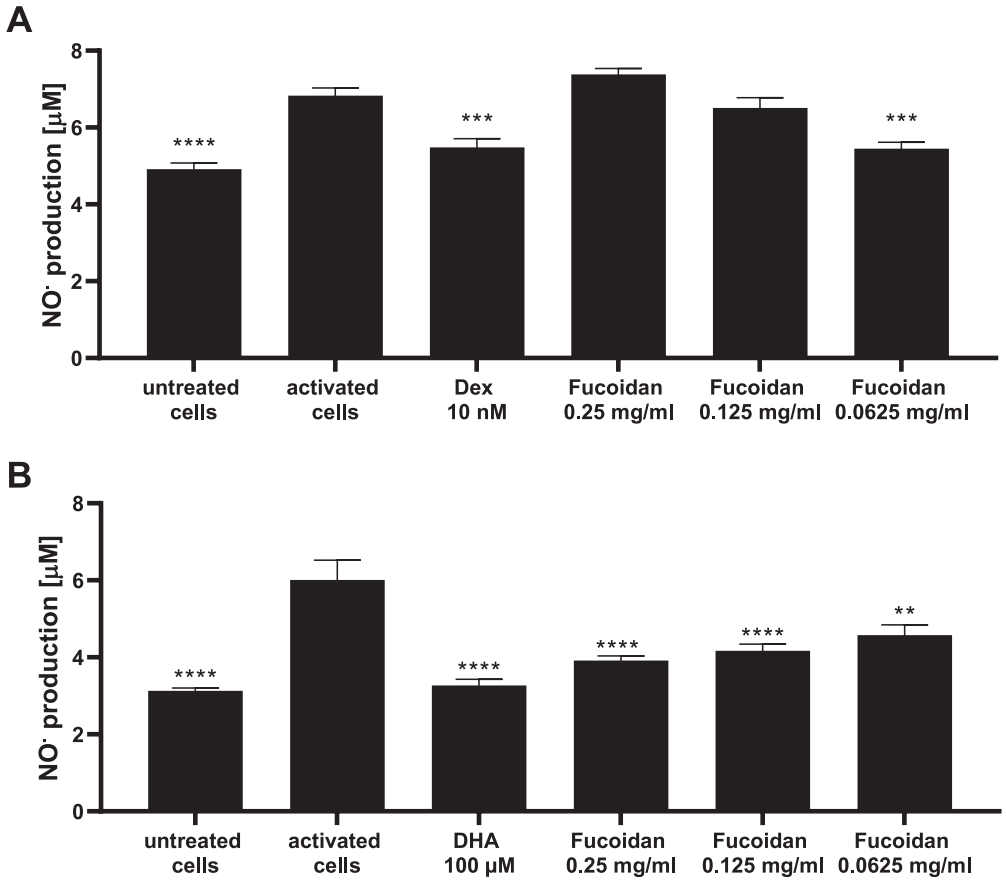

Fig. 7. Effect of tested fucoidan from *Fucus vesiculosus* on the nitric oxide production in PMA-activated HDFa (A) and IFN-γ/LPS-activated J744A.1 (B) cell lines. The data are presented as the mean ± SEM from three independent experiments. Statistical significance in comparison to the activated cells was assessed using one-way ANOVA followed by Dunnett's multiple comparison test; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

3.9. Inhibition of nitric oxide production (Griess assay)

The ability of tested fucoidan to scavenge nitric oxide was determined by the decrease in the absorbance at 550 nm – the result of the reduced production of NO. The potential of tested sample to inhibit nitric oxide production in PMA-stimulated dermal fibroblasts and LPS/IFN-γ-activated murine macrophages are presented in Fig. 7A and 7B, respectively.

As can be seen from Fig. 7A, only the lowest concentration of *Fucus vesiculosus* fucoidan, 0.0625 mg/ml, caused a statistically significant decrease in nitric oxide production. The level of obtained NO was 20 % lower in comparison to the activated cells (PMA-treated HDFa). Observed inhibition of nitric oxide synthesis was close to that obtained after cells treatment with 10 nM dexamethasone, a known anti-inflammatory agent, and a positive control in the experiment. Tested fucoidan showed more effective NO-scavenging activity when was used

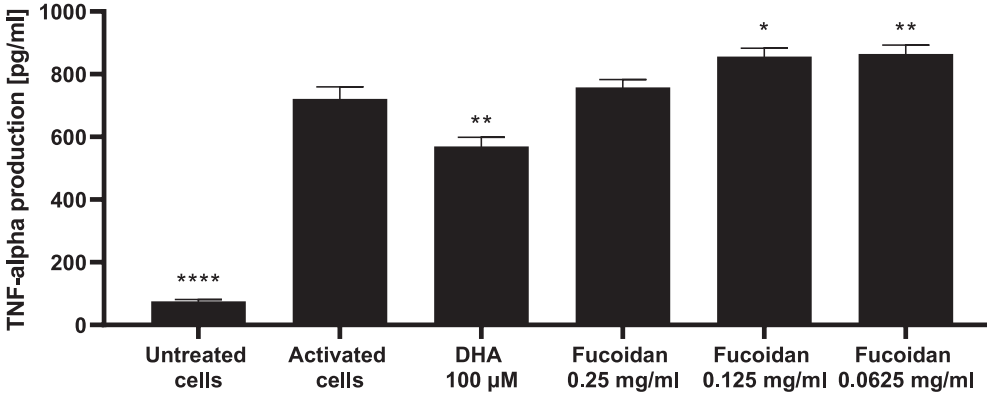

Fig. 8. Effect of tested fucoidan on the TNF-α production in IFN-γ/LPS-activated J774A.1 murine macrophages. The data are presented as the mean ± SEM from three independent experiments. Statistical significance in comparison to the activated cells was assessed using one-way ANOVA followed by Dunnett's multiple comparison test; *$p < 0.05$; **$p < 0.01$;****$p < 0.0001$.

in the mouse macrophages cell culture (Fig. 7B). All tested concentrations of fucoidan protect the skin cells from the pro-inflammatory activity of IFN-γ and LPS. When the tested sample was used at the concentrations of 0.25 mg/ml and 0.125 mg/ml, there were not statistically significant differences between activity of fucoidan and DHA, used as a positive control.

3.10. TNF-α production (ELISA)

Fig. 8 illustrates the results of TNF-α production as a result of the J774A.1 murine macrophages treatment with tested fucoidan isolated from *Fucus vesiculosus*.

100 μM DHA, used as a positive control, was the only sample that caused a statistically significant reduction in TNF-α level in LPS/IFN-γ-activated murine macrophages. Cell treatment with tested fucoidan at concentrations 0.125 mg/ml and 0.0625 mg/ml resulted in statistically significant increase in production of tested inflammatory cytokine.

3.11. IL-12 production (ELISA)

The level of interleukin 12 (IL-12) after murine macrophages treatment with tested fucoidan was measured using ELISA. The obtained results are shown in Fig. 9.

Fucoidan from *Fucus vesiculosus* significantly inhibited production of IL-12 by J774A.1 cell line. There were no differences between the activity of fucoidan used at all three tested concentrations. There was also no significant difference between anti-inflammatory activity of tested sample and positive control, 100 μM DHA.

3.12. IL-6 production (ELISA)

The level of interleukin 6 (IL-6) production by murine macrophages J774A.1 and dermal fibroblasts HDFa treated with tested fucoidan is presented in Fig. 10.

Fucoidan isolated from *Fucus vesiculosus* used at the highest concentration, 0.25 mg/ml, significantly inhibited production of IL-6 by dermal fibroblasts (Fig. 10A). The obtained level of tested interleukin was 77.68 pg/ml, in comparison to the activated cells – 111.15 pg/ml (30 % decrease). Tested fucoidan showed anti-inflammatory activity when tested in murine macrophages J774A.1 (Fig. 10B). All three used concentrations of tested sample caused statistically significant decrease in interleukin-6 production in a dose-dependent manner. The highest inhibition was observed for 0.25 mg/ml fucoidan – the level of obtained IL-6 was 90 % lower in comparison to the activated cells treated with LPS and IFN-γ.

3.13. IL-8 production (ELISA)

The influence of the tested fucoidan on interleukin 8 (IL-8) production by human dermal fibroblasts was measured using ELISA. The results are presented in Fig. 11.

As can be seen from the graph, tested *Fucus vesiculosus* fucoidan significantly increased IL-8 synthesis in skin cell line, HDFa. The inhibition in production of IL-8 was observed only when cells were treated with positive control, dexamethasone (Dex).

4. Discussion

Potential of *Fucus vesiculosus* fucoidan to affect inflammation *in vitro*

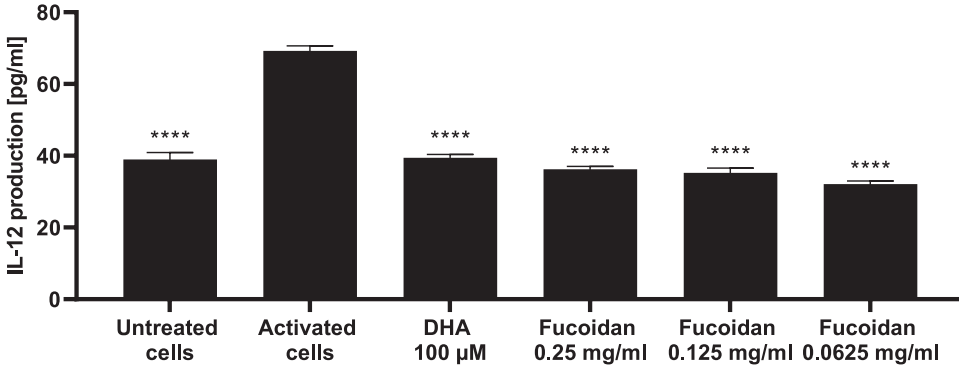

Fig. 9. Effect of tested fucoidan on the IL-12 production in IFN-γ/LPS-activated J774A.1 murine macrophages. The data are presented as the mean ± SEM. Statistical significance in comparison to the activated cells was assessed using one-way ANOVA followed by Dunnett's multiple comparison test; ****$p < 0.0001$.

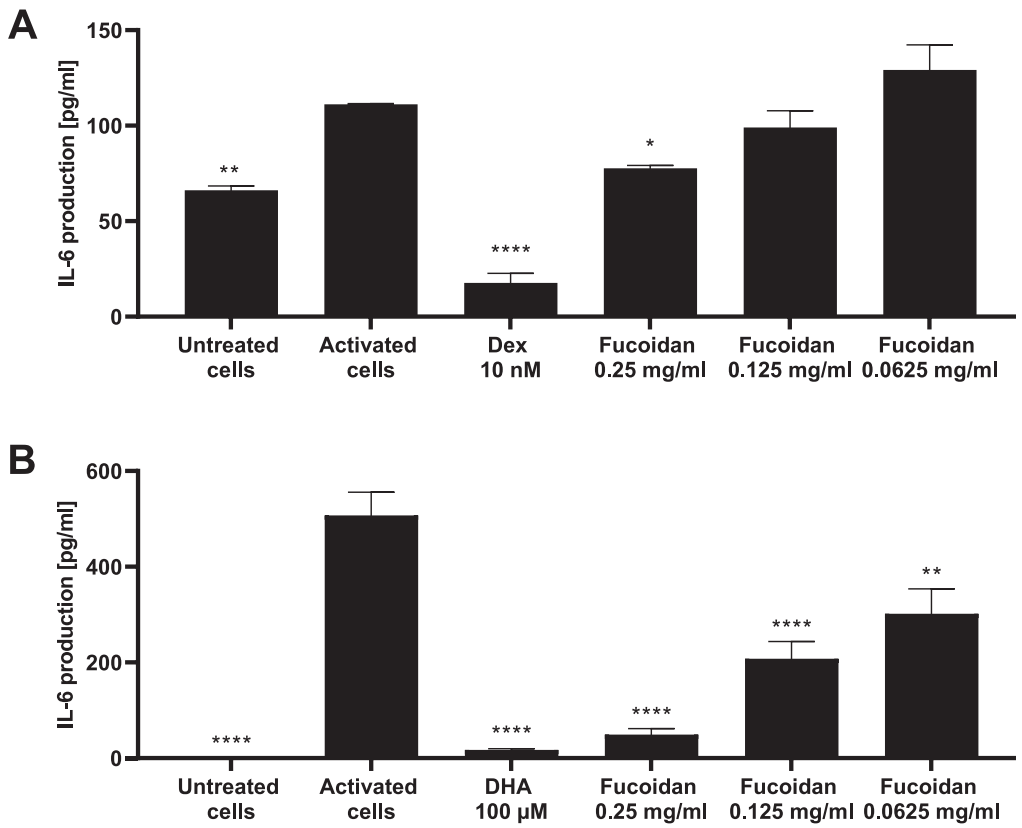

Fig. 10. Effect of tested fucoidan on the IL-6 production in PMA-activated dermal fibroblasts HDFa (A) and IFN-γ/LPS-activated J774A.1 murine macrophages (B). The data are presented as the mean ± SEM. Statistical significance in comparison to the activated cells was assessed using one-way ANOVA followed by Dunnett's multiple comparison test; *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

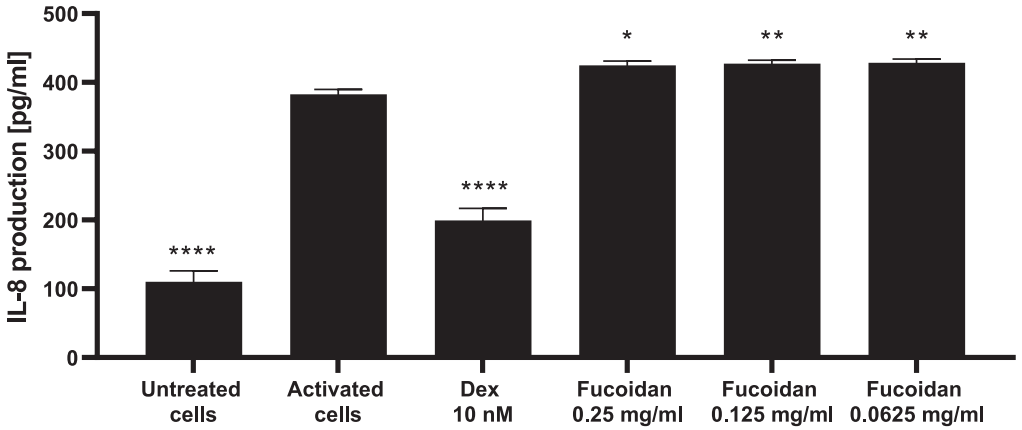

Fig. 11. Effect of tested samples on the IL-8 production in PMA-activated dermal fibroblasts (HDFa). The data are presented as the mean ± SEM from three independent experiments. Statistical significance in comparison to the cells treated with PMA only was assessed using one-way ANOVA followed by Dunnett's multiple comparison test; *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$.

was evaluated in the present study. Literature data indicate that there is a correlation between inflammation and the level of reactive oxygen level [73]. The antioxidant and anti-inflammatory potential of brown seaweed fucoidans were investigated by Obluchinskaya et al. [53]. The strongest antioxidant properties, measured as DPPH scavenging activity, were observed for polysaccharides isolated from *Fucus vesiculosus* (IC50 of 0.5 mg/ml). Fucoidan extracted from the same species also showed the strongest anti-inflammatory effect. Mak et al. [74] analyzed the antioxidant activity of commercially available *Fucus vesiculosus* fucoidan. The sample used at a concentration of 1 mg/ml exhibited DPPH scavenging activity of 55 %. The tested fucoidan used in our study at concentration of 1 mg/ml showed 47 % inhibition of DPPH. The potential of fucoidan to suppress ROS in the present study was confirmed using a cellular antioxidant assay as well. Results obtained from both methods indicate that the tested fucoidan is a potent antioxidant.

Performed in the present study analysis of the NO level after treatment of HDFa and J774A.1 showed that fucoidan reduced release of this inflammation mediator from the cells. In J774A.1 cells, the level of protection against the pro-inflammatory effects of IFN-γ and LPS was similar to that observed after using DHA, a well-known anti-inflammatory agent [75]. Nitric oxide is a signalling molecule which is generated during an immune and inflammatory response by an enzyme, inducible

nitric oxide synthase 2 (NOS-2). This enzyme is not expressed in resting cells but can be induced by pro-inflammatory stimuli such as bacterial lipopolysaccharide [76]. Similar results were obtained by Lee et al. [77]. Nitric oxide produced in murine macrophages RAW 264.7 as a result of treatment with LPS was significantly inhibited by the addition of three fractions of fucoidan extracted from *Eclonia cava* (0.05–0.1 mg/ml). Anti-inflammatory activity of fucoidan via reduction of NO production was also proved on the genetic level. The polysaccharide isolated from *Eclonia cava* inhibited, in dose dependent manner, the enzyme iNOS and the gene expression for the cyclooxygenase-2 (COX-2) and, consequently, the production of nitric oxide and prostaglandin E2 in LPS-treated RAW 264.7 cells [78]. Park et al. [37] observed an inhibition in iNOS expression in RAW 264.7 cells activated by LPS after treatment with 16 μg/ml fucoidan isolated from *Fucus vesiculosus*. Polysaccharide from the same seaweed species decreased secretion of PGE1, TNF-α and IL-1β in LPS-activated RAW 264.7 cells. It was concluded that fucoidan suppress early stage of inflammation and its activity is related to the NF-κB signalling pathway, as tested compound prevented the accumulation of NF-κB p65 subunit in the nucleus [39]. Anti-inflammatory property of fucoidan was also reported by Amin et al. [36]. Tested sample reduced nitric oxide level and expression of CD86 in LPS-treated murine RAW 264.7 macrophages. The activity of fucoidan was compared by the authors to the effect of IL-10, the anti-inflammatory cytokine. Treatment of LPS-activated RAW 264.7 cells with fucoidan resulted in the inhibition of NO synthesis and the inhibition in production of pro-inflammatory mediators (TNF-α, IL-1β and IL-6) when tested compound was isolated from *Saccharina japonica* [79], *Ecklonia cava* [77] and *Sargassum horneri* [80]. Our results are consistent with these findings. Inhibition of IL-6 synthesis by human dermal fibroblasts and mouse macrophages was also observed in the present study. The anti-inflammatory effect of fucoidan was especially observed in murine macrophages cell model – tested samples significantly reduced IL-6 production when used at all three tested concentrations.

However, some researchers reported also that fucoidan can increase production of pro-inflammatory cytokines and act as a macrophage activator [81–87]. Polysaccharide used in the present study also showed pro-inflammatory activity. It was observed that the tested sample significantly increased the level of TNF-α in murine macrophages and the level of IL-8 in human dermal fibroblasts. According to the Yang et al. [81] fucoidan used at low concentration – 10 μg/ml, increased the expression of iNOS in quiescent macrophages. The same compound used as a stimulant of LPS-activated macrophages RAW 264.7 caused a statistically significant inhibition in iNOS induction. It was experimentally proven that induction of iNOS was related to selective suppression of AP-1 activation (activator protein 1) [81]. Leiro et al [82] analyzed immunomodulating properties of fucoidan extracted form *Ulva rigida*. It was reported that ulvan polysaccharides stimulated RAW 267.4 cells and induced the secretion of PGE2 and NO through the expression of iNOS and COX2 mRNAs. Fucoidan extracted from *Laminaria angustata* var. *longissima* used at concentrations between 50 and 400 μg/ml caused a statistically significant increase in NO generation by murine cells RAW 264.7. Higher production of nitric oxide was correlated with an increase in iNOS expression measured by immunoblot analysis. Fucoidan was the only compound used for cell stimulation. The researchers observed also increases in cytokines, TNF-α and IL-6 in murine macrophages treated with the tested fucoidan for 24 h in a dose-dependent manner [83]. Zhang et al. [87] evaluated influence of fucoidans isolated from four seaweed species (*Ascophyllum nodosum*, *Macrocystis pyrifera*, *Undaria pinnatifida* and *Fucus vesiculosus*) on human neutrophils. A significant increase in the production of IL-6, IL-8 and TNF-α was detected. Similar observations were made by Tabarsa et al. [85]. Fucoidan from *Nizamuddinia zanardinii* caused an increase in the production of nitric oxide, TNF-α, IL-1β and IL-6 from murine macrophages RAW 264.7. It was stated that tested cells were activated through two signalling pathways, NF-κB and MAPK. Fucoidan extracted from *Agarum cribrosum* stimulated RAW 264.7 macrophages through the activation of NF-κB and MAPKs pathways, which resulted in the generation of nitric oxide and the induction of mRNA expression of cytokines [88]. Literature data suggest also that fucoidan isolated from *Undaria pinnatifida* induced chemokine production (RANTES and MIP-1α) from RAW 264.7 cells [89]. MIP-1 α is a known inducer of the synthesis and releasing of pro-inflammatory cytokines from fibroblasts. Immunomodulatory activity of fucoidan extracted from various species of seaweed was proven in different *in vitro* studies. Yung-Choon et al [89] tested the immunomodulating activity of fucoidans from *Fucus vesiculosus* and *Undaria pinnatifida* on two types of murine cells: RAW 264.7 and peritoneal macrophages. They observed that treatment of fucoidan induced TNF-alpha in RAW 264.7 cells, and the level of cytokine was higher after using *Undaria pinnatifida* polysaccharide. In contrast to TNF-α generation, fucoidan from *Fucus vesiculosus* showed higher activity in production IL-6. Both fucoidans did not induce IFN-γ. It was reported that fucoidan from *Sargassum fusiforme* induced TNF-α and IL-1β secretion from murine macrophages [90]. It seems that activation of macrophages by fucoidan may be related to the receptors present on the cell surface. Fucoidan is a ligand for a scavenger receptor A (SR-A, CD204) on macrophages. Yu et al. [91] investigated whether SR-A cooperates will Toll-like receptors (TLRs) in response to TLR ligand stimulation (e.g. LPS). In the study, murine macrophages J774A.1 were treated with different ligands in the presence or absence of fucoidan. The results indicate that fucoidan synergized with LPS to enhance NF-κB activation and expression of inflammatory genes. To confirm these data, researchers isolated peritoneal macrophages from SR-A-, TLR4-deficient mice and wild type mice. Cells were treated with LPS with or without tested fucoidan. LPS-stimulated TNF-α and IL-1β production was further enhanced in the wild type macrophages when fucoidan was present. The results suggest that SR-A is needed for LPS-stimulated inflammatory response in macrophages [91]. According to Nakamura et al. [92], fucoidan-stimulated production of nitric oxide is also dependent on the using of SR-A route.

The assessment of biological activities of fucoidan cannot be performed in isolation from its structure and chemical composition. Used in the present study, *Fucus vesiculosus*-derived fucoidan, although of purity 97 %, is a mixture of both carbohydrate and non-carbohydrate components (Table 1). The latter elements, which are co-extracted with fucoidan, may influence the observed biological effects. The anti-inflammatory and immunomodulatory activity of fucoidan has been correlated with uronic acid content. Polysaccharide isolated from *Undaria pinnatifida*, with uronic acids content of around 1 %, increases the level of IFN-γ with no significant alterations in the production of of IL-4, IL-6, TNF-α, and NF-κB [93]. In turn, fucoidan with a higher content of uronic acids (one glucuronic acid residue for every 6 mol of fucose), extracted from *Cladosiphon okamuranus* Tokida decreases IL-6 expression and inhibits activation of the NF-κB signaling pathway in LPS-stimulated murine colonic epithelial cell line [94]. Fucoidan derived from *Cystoseira crinite* which contains 13 % of uronic acids, significantly decreases serum levels of IL-1β, but not IL-10, in rats [95]. It was reported that the influence of fucoidan on the immune system is related to the content of sulphate groups. Wu et al. reported that fucoidans isolated from *Sargassum cristaefolium*, with different molecular weights or sulfate contents exhibited different anti-inflammatory effect [96]. Polysaccharide with 9.4 % sulphate content was characterized by higher NO inhibitory activity in RAW264.7 cells activated with LPS. The authors suggested that the sulphate content affects fucoidan binding with the cell receptor and the production of nitric oxide. The bioactivity of fucoidan is also correlated with its molecular mass [97,98]. It was observed that fucoidan molecules with lower molecular mass more easily pass through the cellular membrane and showed stronger biological effects in comparison to the high molecular weight fucoidans [98]. Fucoidan ability to pass the membrane (active transport) is an important factor in evaluating its anti-inflammatory or immunomodulatory activity, especially under *in vivo* conditions, where bioavailability of tested samples is crucial [99–101].

5. Conclusion

It can be concluded that many factors may influence the biological activity of fucoidans. Seaweed-sourced polysaccharides exert various anti-inflammatory effects depending on the source, target cell, and injury model. The structure of fucoidans, molecular weight and sulphate heterogenicity, especially the degree of sulphation, position of the sulphate moiety and organization of sulphated domains, may play a critical role in fucoidan biological effects. Immunomodulatory activity of sulphated polysaccharides has been demonstrated in many studies and results indicate that fucoidans are potent immunostimulants. In the present study, both anti-inflammatory and pro-inflammatory properties of *Fucus vesiculosus* fucoidan were observed. Tested samples showed antioxidant activity when tested in non-cellular and cellular assays. The obtained results, together with the data from NO assay and IL-6 and IL-12 ELISA (HDFa and J774A.1) might be an indicator of fucoidan ability to suppress inflammation.

On the other hand, fucoidan caused increased production of IL-8 by dermal fibroblasts and TNF-α from mouse macrophages, what suggest pro-inflammatory activity of tested sample.

A reduction in nitric oxide or inhibition of cytokines production may be a valuable tool to fight chronic inflammation. The ability of fucoidan to activate macrophages may contribute to their effectiveness in the immunoprevention of cancer or other diseases where macrophage function is impaired or needs to be boosted. Despite the potential of fucoidan for the treatment of inflammation-related conditions, it is necessary to validate the *in vitro* data in human trials. Moreover, it should be emphasized that all results were obtained after using commercially available *Fucus vesiculosus* fucoidan (Sigma Aldrich Co. Ltd.), with a specific lot number and purity of 97 %. The compounds that are extracted simultaneously with fucoidan affect its purity and negatively influence the reproducibility of the results. For this reason, it is crucial to continue research on the biological activity of fucoidan and on the correlation between its chemical structure and biological properties. Future work should also focus on fucoidan purification, to eliminate the influence of other co-extracted molecules on observed biological activity.

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CRediT authorship contribution statement

Aleksandra Augustyniak: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Joanna Przyborska:** Methodology, Investigation, Formal analysis, Data curation. **Helena McMahon:** Writing – review & editing, Validation, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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