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Study of fucoidans as natural biomolecules for therapeutical applications in osteoarthritis

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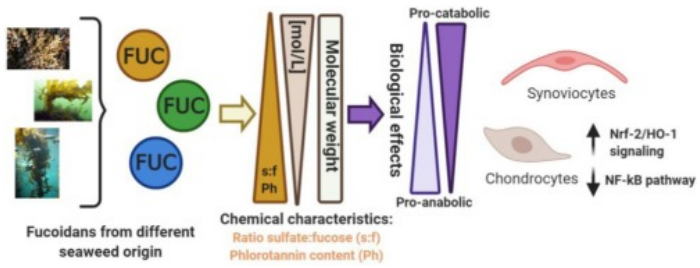
Abstract

Osteoarthritis (OA) is the most prevalent articular chronic disease. Although, to date there is no cure for OA. Fucoidans, one of the main therapeutic components of brown algae, have emerged as promising molecules in OA treatment. However, the variability between fucoidans makes difficult the pursuit of the most suitable candidate to target specific pathological processes. By an in vitro experimental approach in chondrocytes and fibroblast-like synoviocytes, we observed that chemical composition of fucoidan, and specifically the phlorotannin content and the ratio sulfate:fucose, seems critically relevant for its biological activity. Nonetheless, other factors like concentration and molecular weight of the fucoidan may influence on its beneficial effects. Additionally, a cell-type dependent response was also detected. Thus, our results shed light on the potential use of fucoidans as natural molecules in the treatment of key pathological processes in the joint that favor the development of rheumatic disorders as OA.

Graphical abstract



Abbreviations

OA, osteoarthritis; FLS, fibroblast-like synoviocytes; NO, nitric oxide; iNOS, isoform of NO synthase; IL, interleukin; PGs, prostaglandins; COX-2, inducible isoform of cyclooxygenase; NF-κB, nuclear factor kappa B; Nrf-2, nuclear factor (erythroid-derived 2)-like 2; FF, fucoidan from Fucus vesiculosus; FM, fucoidan from Macrocystis pyrifera; FU, fucoidan from Undaria pinnatifida; RI, refractive index; TCA, trichloroacetic acid; BSA, bovine serum albumin; TEAC, trolox equivalent antioxidant capacity; HPSEC, high performance size exclusion chromatography; FTIR, fourier-transform infrared spectroscopy; ELISA, enzyme-linked immunosorbent assay; MTT3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; DMEM, dulbecco's modified Eagle's médium; FBS, fetal bovine serum; AA, antimycin A; TMRM, tetramethylrhodamine methyl ester; ΔΨm, mitochondrial membrane potential; DCFDA, 2',7'-diclorodihidrofluoresceína; PMSF, phenylmethylsulfonyl fluoride; HO-1, heme oxygenase-1; SEM, standard error of the mean; MRC, mitochondrial respiratory chain

Chemical compounds studied in this article

Fucoidan (PubChem CID: 92023653); Fucose (PubChem CID: 3034656); Galactose (PubChem CID: 6036); Glucose (PubChem CID: 5793); Potassium sulfate (PubChem CID: 24507); Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (PubChem CID: 40634); Phloroglucinol (PubChem CID: 359); Dextran (PubChem CID: 4125253); Interleukin-1 beta (PubChem CID: 123872); Antimycin A (PubChem CID: 14957)

Keywords

Fucus vesiculosus fucoidan; Macrocystis pyrifera fucoidan; Undaria pinnatifida fucoidan; Osteoarthritis; Osteoarthritic chondrocytes; Synovial fibroblasts

1. Introduction

Rheumatic diseases are a heterogeneous group of disorders mainly affecting the joint. Some of these disorders are among the most common diseases worldwide. Likewise, osteoarthritis (OA) is the most prevalent articular chronic disease, occurring in 10–20 % of the population over 50 years of age (Vaamonde-García & López-Armada, 2019). The global prevalence of hip and knee OA is approaching 5 % and is expected to rise up as the population ages (Kraus, Blanco, Englund, Karsdal, & Lohmander, 2015; Vaamonde-García & López-Armada, 2019). However, there is no cure for OA, thus this pathology is managed rather than cured, with a focus on alleviating its pain and attenuating its progression.

The pathogenesis of OA mainly involves cartilage degradation, subchondral sclerosis and synovial inflammation, in turn causing pain and loss of articular function (Kraus et al., 2015). Chondrocyte is the unique cell type found in the articular cartilage and responsible in the maintenance and regeneration of extracellular matrix of this tissue. Nowadays, it is widely accepted that the disruption in the balance between catabolic and anabolic processes in the chondrocyte contributes to cartilage destruction in the OA (Kraus et al., 2015; Robinson et al., 2016). Mitochondria play an important role in the chondrocyte metabolism and subsequently in cartilage homeostasis. Hence, alterations in mitochondrial function have been associated to pathological events taking place in OA, such as increased oxidative stress and cell death, and up-regulation of pro-inflammatory cytokine production (Vaamonde-García & López-Armada, 2019). In addition to chondrocytes, fibroblast-like synoviocytes (FLS) from fluid and synovial membrane also show up-regulated synthesis of catabolic mediators in the OA joint that in turn amplifies joint inflammation, setting a vicious circle that favors to OA onset (Robinson et al., 2016; Vaamonde-García & López-Armada, 2019).

Nitric oxide (NO) is an endogenously produced gas with physiological functions in the joint (Wahl et al., 2003). However, excessive production of this gas by up-regulated synthesis of inducible isoform of NO synthase (iNOS) could activate catabolic events responsible for participating in OA pathogenesis, including mitochondrial dysfunction, the expression of proinflammatory cytokines like interleukin 6 (IL-6) and prostaglandins (PGs) (Abramson, 2008). Interleukin 6 is a pivotal cytokine involved in synovial inflammation and mechanisms underlying chronic pain, among other processes occurring in OA (Lin, Liu, Jiang, Zhou, & Tang, 2017). PGs are pivotal factors in inflammatory processes that are synthetized by cycloxygenase and PG synthase enzymes. The expression of inducible isoform of cyclooxygenase, COX-2, is triggered by oxidative stress and pro-inflammatory mediators like interleukin 1β (IL-1β) (Amin, Dave, Attur, & Abramson, 2000; Lepetsos, Papavassiliou, & Papavassiliou, 2019). IL-1 signaling plays a central role on the different cell types involved in OA, and could represent attractive target for the development of novel drugs in the treatment of this pathology (Jenei-Lanzl, Meurer, & Zaucke, 2019; Wojdasiewicz, Poniatowski, & Szukiewicz, 2014). IL-1β mediates in the downregulation of components of extracellular matrix synthesis as well as in the upregulation of pro-inflammatory mediators, including IL-6, PGE2, COX-2, or iNOS and NO (Jenei-Lanzl et al., 2019; Wojdasiewicz et al., 2014). Likewise, the activation of transcriptional factor NF-κB, nuclear factor kappa B, by IL-1β is responsible of a large part of these catabolic effects (Jenei-Lanzl et al., 2019; Lepetsos et al., 2019; Wojdasiewicz et al., 2014).

Natural biomolecules have gained a great interest within food and non-food sectors for its healthy properties such as antioxidant, anti-inflammatory, antiobesity, antitumoral and other related with the health (Tiwari & Declan, 2015). Marine resources could be attractive alternatives to cope the growing demand by natural biomolecules (Prameela, Mohan, & Ramakrishna, 2018). Namely, bioactive compounds from seaweeds have a great potential for biomedical applications. Different environmental factors can influence on the composition of the seaweeds as geographical location or collection season. Algae are divided according to their pigmentation in green, red or brown, being the majority pigment chlorophyll, phycobilins and fucoxanthins, respectively. Minerals, vitamins, lipids, fatty acids and other compounds are also present in seaweeds as minor compounds and also have an important role as bioactive compounds (Hamid et al., 2015).

Particularly, brown seaweeds contain sulfated polysaccharides with biological potential, renowned as fucoidan. It is comprised mainly by fucose and sulfate groups, nevertheless, other compounds are present in their structure as glucose, mannose, rhamnose or acetyl groups. Several factors have influence in the composition, structure and biological properties of the fucoidans (Rodrigues et al., 2015). According to literature, its biological activity is associated with the molecular weight, structure and sulfate groups, depending on their content and sulfate positions, the fucoidan can be more active (Chen et al., 2019). The structure-activity relationships of fucoidan have been addresses in different studies (Cong et al., 2016; Koh, Lu, & Zhou, 2019; Liu et al., 2017). Geographical location, season collection, nutrients and other abiotic factors directly have influenced in their composition and, thus, in their structure. Besides, the extraction technology used to achieve this sulfated polysaccharide is another key factor (Ale & Meyer, 2013; Ale, Mikkelsen, & Meyer, 2011). Most of the recent therapeutic applications using fucoidans have used commercial products, especially those from Fucus vesiculosus and Undaria pinnatifida (Bittkau et al., 2019; Lu et al., 2018), which belong to Fucales and to Laminarales, respectively. For instance, the antiproliferative effect of commercial fucoidan from Fucus vesiculosus was evaluated in tumoral and non-tumoral cell lines (Bittkau et al., 2019). The clinical use of fucoidans has been encouraged in pathologies such as cancer, neurological diseases and diabetes (H. J. Fitton, Stringer, Park, & Karpiniec, 2019). Likewise, there is a growing number of studies suggesting a protective impact of fucoidans in rheumatic disorders (J. H. Fitton, 2011). Nonetheless, evidences supporting the application of these polysaccharides in OA treatment are still scarce, and more studies are necessary to further underpin its future therapeutic use. Fucoidans exhibited broad bioactivities, including antitumoral, anti-inflammatory and antioxidant properties. These activities rely on a variety of cellular and molecular mechanisms such as inhibition of interaction of selectins and PGs, down regulation of cytokines and chemokines, inhibition of metalloproteinases, reduction of oxidative stress, and modulation of activities of transcription factors like NF-κB and nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) (Phull & Kim, 2017a, 2017b; Ryu & Chung, 2016). A representative structure of fucoidan from F. vesiculosus, M. pyrifera and U. pinnatifida is presented in Fig. 1. The duality of fucoidan acting as an anti-inflammatory and proinflammatory agent has also been associated to this variability, so that disparity between composition, molecular weight and the source species make difficult to establish comparisons between the actions of these polysaccharides in the different studies (Flórez et al., 2017).

Overall, recent findings suggest that fucoidans are promising candidates to address the symptoms of OA, however its use is still insufficiently supported by scientific research. In this study we evaluated the protective effect of different fucoidans on catabolic pathways activated in articular cells. To achieve this aim, we first analyzed the structure and composition of fucoidans from Fucus vesiculosus, Macrocystis pyrifera and Undaria pinnatifida, and then chondrocytes and FLS were treated with them in order to evaluate and compare the effect of these polysaccharides on pathological pathway activated in articular cells by different catabolic stimuli.

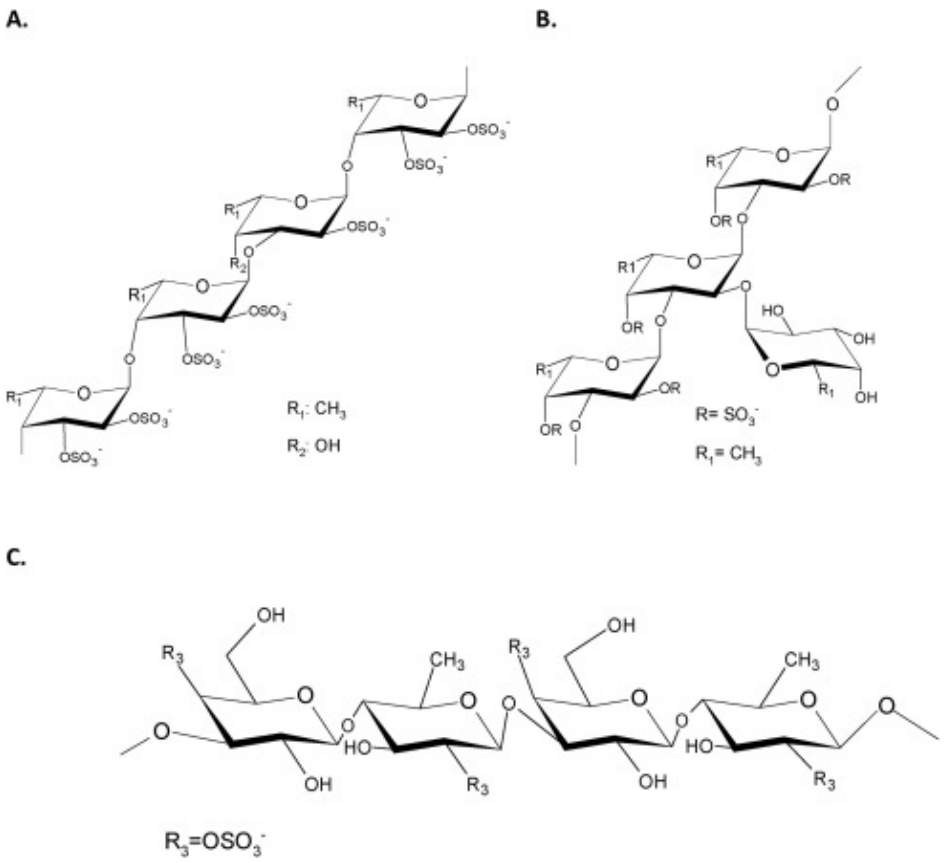


Fig. 1. Illustrative base structure of fucoidans from Fucus vesiculosus (A), basic structure of the order Laminariales as a representative pattern of Macrocystis pyriferia (B), and Undaria pinnatifida (C) [adapted from (Ale, Maruyama, Tamauchi, Mikkelsen, & Meyer, 2011; Koh et al., 2019; Patankar, Oehninger, Barnett, Williams, & Clark, 1993)]. Note here that the structures were graphed using ACD/Labs Chemsketch software version 11.02 (Advanced Chemisty Development, Toronto, Canada).

2. Material and methods

2.1. Materials and characterization

Fucoidans from the seaweed Fucus vesiculosus L. (FF), also known as bladder fucus and/or rockweed, Macrocystis pyrifera L. (FM), common name giant kelp, and Undaria pinnatifida Harvey (FU), known as brown kelp, were purchased to Sigma-Aldrich. Fucose, galactose, glucose, formic acid, acetic acid, ABTS (2, 2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), trichloroacetic acid (TCA), BaCl2, Bradford reagent, bovine serum albumin (BSA), KBr and phloroglucinol also from Sigma-Aldrich (Spain). Folin-Ciocalteu (2 N), sodium carbonate (Na2CO3), gelatin powder, potassium sulfate (K2SO4) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased in Scharlau (Spain). Dextrans from Fluka (USA).

2.1.1. Oligosaccharides content

The analysis of oligosaccharides was performed, previously a hydrolysis using sulfuric acid (4 %) was necessary where 10 mL of fucoidan sample with 0.4 mL of sulfuric acid were placed in a closed pyrex bottle, and introduce in an autoclave (MED 12, P-Selecta, Spain) at 121 °C for 20 min. The samples were cooled until room temperature (25 ± 2 °C) and filtered through 0.45 μm membranes (Sartorius, Spain). The determination of glucose, galactose, fucose, formic acid and acetic acid was performed by HPLC (1100 series, Agilent, Germany) using these compounds as patterns. The equipment was provided with a refractive index (RI) working at 35 °C, the column used for this determination was Aminex HPX-87H (BioRad, USA) working at 60 °C. The operational conditions for the mobile phase were a flow rate at 0.6 mL/min using H2SO4 at 0.003 M.

2.1.2. Sulfate content

Following the protocol described for the determination of sulfate content (Dodgson, 1961). Samples of fucoidans were evaluated by the Gelatin-BaCl2 method. At first, the main reagent (gelatin-BaCl2) was prepared dissolving 0.5 g of gelatin powder in 100 mL of distilled, that is 0.5 % (w/v) in hot water (70 °C), after kept at 4 °C for 12 h. Afterwards, 0.5 g BaCl2 was added to gelatin solution to obtain a cloudy solution, after 2−3 h the gelatin-BaCl2 reagent is ready to use. Shortly, 1.9 mL of TCA 4% (4 g of TCA in 100 mL of distilled water) was added above 0.1 mL of sample in a test tube, next 0.5 mL of gelatin-BaCl2 reagent was added, and mixed in a vortex. A brief incubation at room temperature (25 ± 2 °C) for 15 min was required. The standard curve was performed with potassium sulfate (1.813 g K2SO4 in 100 mL of distilled water), and the absorbance was measured at 500 nm in a spectrophotometer (Evolution 201 UV–vis, Thermo Scientific, USA). The assay was performed at least in triplicate.

2.1.3. Soluble protein content

The assay was based on the protocol described by Bradford method (Bradford, 1976). In this context it was necessary to follow the instructions provided by Sigma-Aldrich for the Bradford reagent. The samples (0.1 mL) were introduced in a test tube, after Bradford reagent was added (1 mL) above and mixed in a vortex, to get a complete reaction between the reagent and the sample. BSA was used to prepare the standard curve. The absorbance of samples and BSA dilutions were measured, at least in triplicate, at 595 nm in a spectrophotometer (Evolution 201 UV–vis, Thermo Scientific, USA).

2.1.4. Phloroglucinol content

The quantification of phloroglucinol content was performed following the protocol detailed henceforth (Koivikko, Loponen, Honkanen, & Jormalainen, 2005). Fucoidan samples and distilled water for the blank (1 mL) were placed in a test tube, Folin-Ciocalteu 2 N (dilution 1:1 with distilled water to achieve concentration 1 N) was added above (1 mL) and Na2CO3 20 % (2 mL) was also added and mixed with a vortex (ZX3, VELP Scientifica, Italy). Afterwards, incubation period for 45 min at room temperature (25 ± 2 °C) was necessary. Phloroglucinol was used as a pattern to perform the standard curve. Samples, blank and patterns were read at 730 nm, at least in triplicate, in a spectrophotometer (Evolution 201 UV–vis, Thermo Scientific, USA).

2.1.5. Antioxidant assay

Trolox equivalent antioxidant capacity (TEAC) value was determined using the ABTS radical scavenging method (Re et al., 1999). Based on a spectrophotometric measured, TEAC reagent was prepared (34.8 mg of ABTS and 6.62 mg of potassium persulfate dissolved in 10 mL of PBS, the solution was stir for 16 h in darkness), in order to use, firstly, the TEAC solution have been equilibrated at 30 °C and diluted with PBS (also used as blank) until achieve adjust the absorbance to 0.7 (measuring at 734 nm). Samples or pattern (20 µL) and diluted TEAC reagent (2 mL) were mixed and incubated at 30 °C for 6 min, PBS was used as a blank. Trolox pattern was used to prepare the standard curve. The absorbance of the samples was measured at 734 nm (Evolution 201 UV–vis, Thermo Scientific, USA), at least in triplicate.

2.1.6. Molar mass distribution

High performance size exclusion chromatography (HPSEC) was used to study the molar mass distribution of the samples. The determination was performed by HPLC (1100 series, Agilent, Germany), the equipment was provided with a refractive index (RI) working at 35 °C, with two columns in series TSKGel G3000PWXL and TSKGel G2500PWXL (300 × 7.8 mm) and a PWX-guard column (40 × 6 mm). The operation conditions were: the mobile phase at 0.4 mL/min with Milli-Q water and the column module working at 70 °C. Dextrans (DX) were used as patterns (80, 50, 25, 12, 5 and 1 kDa). The measured was performed at least in duplicate.

2.1.7. Fourier-transform infrared spectroscopy (FTIR)

Samples of commercial fucoidans from Fucus vesiculosus, Macrocystis pyrifera and Undaria pinnatifida were blended with KBr, to prepare the sample under the specification of the equipment, and analyzed by FTIR. The equipment used was Nicolet 6700 (Thermo Scientific, USA), the source was IR, the detector DTGS KBr and the software: OMNIC. The FTIR spectra were obtained with a spectral resolution of 4 cm−1 (32 scans/min) and the range was from 400 nm to 4000 nm. The assay was carried out in duplicate.

2.2. Cells culture and treatment

OA human chondrocytes and FLS were obtained as previously described from the knee or hip joints of 8 adult donors (mean ± SD age 71 ± 13 years; n = 3 men and 5 women) and 7 adult donors (mean ± SD age 79 ± 12 years; n = 2 men and 5 women) with osteoarthritis, respectively (Vaamonde-García et al., 2012; Vaamonde-García et al., 2019). Subcultures of isolated cells from cartilage and synovium were performed with trypsin-EDTA (Gibco Life Technologies, UK), after first-passage chondrocytes were used for experiments, whereas FLS were used between third- and eighth-passage. Cells were seeded into 12-well plates (Corning Costar, USA) for protein and flow cytometric analysis, 96-well plates (Costar) for enzyme-linked immunosorbent assay (ELISA) of IL-6 and PGE2, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, and NO measurement, or 8-well chamber slides (Becton Dickinson) for immunocytochemistry studies. When cells reached confluence, they were made quiescent by 48-hour incubation in Dulbecco's modified Eagle's medium (DMEM) (Gibco Life Technologies) containing 0.5 % fetal bovine serum (FBS; Gibco). After washing, the experiments were performed without FBS for chondrocytes and with 0.5 % FBS for FLS. Primary cultured cells were treated with FF, FM, and FU at 5, 30 and 100 μg/mL based on previous literature (Kim & Lee, 2012; Ryu & Chung, 2016; Shu, Shi, Nie, & Guan, 2015). To activate inflammatory pathways, cells were stimulated with IL-1β (5 ng/ ml; Sigma-Aldrich). Antimycin A (AA) (Sigma-Aldrich) were employed as inhibitor of mitochondrial respiratory chain complexes III (Vaamonde-García et al., 2012). All studies were performed strictly in accordance with current local ethics regulations and declaration of Helsinki. Likewise, informed consent was obtained for experimentation with human samples.

2.3. MTT viability assay

Synovial fibroblasts were grown in 96-well plates in DMEM supplemented with 10 % FBS. The cells were then made quiescent by two days' incubation in medium containing 0.5 % FBS. Subsequently, the cells were treated with different stimuli in DMEM for 48 h. Cell viability was evaluated by the measurement of enzymatic reduction of MTT to its insoluble formazan using MTT Cell Assay Kit (Sigma-Aldrich). Then, crystals were dissolved using a solubilization solution and the resulting colored solution quantified by measuring absorbance at 500–600 nanometers using a multi-well spectrophotometer. The relative cell viability was represented by the percentage of absorbance in each experimental condition in relation to those values obtained in basal condition (100 %).

2.4. Measurement of mitochondrial membrane potential

In order to induce a mitochondrial membrane depolarization, chondrocytes were stimulated with AA 0.5 μg/mL in the presence or absence of fucoidans. Cells were loaded with the fluorescent probe tetramethylrhodamine methyl ester (TMRM; Molecular Probes, USA) for the last 30 min of incubation. The TMRM was used in the non-quenching mode (25 nM), so that polarized mitochondria accumulate more fluorescent dye, whereas depolarized mitochondria (lower mitochondrial membrane potential [ΔΨm]) retain less dye and, hence, show lower fluorescence intensity. AA 10 μg/mL served as a positive control for mitochondrial depolarization. The fluorescent signal was monitored using a flow cytometer.

2.5. Measurement of ROS production

2',7'-diclorodihidrofluorescein (DCFDA) and MitoSOX™ (Thermo-fisher, USA) were used to evaluate the intracellular and mitochondrial production of ROS, respectively. The dyes diffuse through the cell membrane and react with ROS and mitochondrial superoxide to generate highly fluorescent compounds. Chondrocytes were stimulated with AA in the presence or absence of fucoidans as previously indicated for 2 h (DCFDA) or 1 h (MitoSOX™), and fluorescence probes were added for the last 30 min of incubation. Then, cells were washed with PBS, collected with trypsin-EDTA, centrifugated, and subsequently resuspended in PBS. Fluorescence intensity was analyzed by flow cytometry in the fluorescence channel 1 (DCFDA) or fluorescence channel 2 (MitoSOX™), and expressed as median fluorescence intensity.

2.6. Western blot

Cells were lysed with Tris-HCl buffer pH 7.5 containing protease and phosphatase inhibitors cocktail (25 mM β-glycerophosphate, 1 mM Na3VO4, and 1 mM NaF) and phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich), and total proteins were separated by SDS-PAGE as previously described (Vaamonde-García et al., 2019). Membranes were incubated with mouse anti-human COX-2 (1.100) and iNOS (1.250) (R&D Systems, Germany), and rabbit anti-human Nrf-2 (1.100) (Santa Cruz Biotechnology, Germany) and anti-human heme oxygenase-1 (HO-1; 1.1000) (Enzo Life Sciences, Lausen, Switzerland) antibody for 16 h at 4 °C. The binding of antigen-antibodies was visualized with 1:1000-diluted anti-mouse or anti-rabbit (Dako, Germany) secondary antibodies and ECL chemiluminescent reagents (Millipore, USA). The ImageQ image processing software (http://imagej.nih.gov/) was used to quantify the protein bands by densitometry. The band intensity of targeted protein was calculated by its related tubulin band intensity for the normalization process.

2.7. IL-6 and PGE2 assays

The levels of IL-6 and PGE2 in culture supernatants from cultured chondrocytes and FLS were determined using commercially available ELISA duo set kit for IL-6 (R&D system) and EIA PGE2 (Cayman, USA) according to the recommendations of the manufacturers. Cells were seeded in 96-well plates and stimulated in 100 μL of DMEM for 24 or 48 h for FLS or chondrocytes, respectively. Data are expressed as picograms released per mL. The working range was between 9.38 and 600 pg/mL for IL-6 and between 7.8 and 1000 pg/well for PGE2.

2.8. Immunofluorescence

Articular cells were fixed in acetone for 10 min at 4 °C and then washed three times in PBS. Subsequently, samples were blocked in PBS-0.2 % Tween 20 (PBST) + 2 % BSA with 4 % Triton-X for 10 min, and incubated with mouse anti-human NF-κB (Santa Cruz Biotechnology) antibody for 16 h at 4 °C. The wells were then washed with PBST and FICT-labeled rabbit anti-mouse secondary antibody (DAKO A/S) was incubated for 1 h. Cell nuclei was then counterstained with DAPI and examined using an inverted microscope CKX41 (Olympus, Belgium). ImageJ was used to measure the percentage of positive area among the articular cells.

2.9. Statistical analysis

One-way ANOVA analysis was performed with the experimental data in Table 1 using the software Statistica (version 10.0, StatSoft Inc., USA). Whenever the analysis exhibited differences between means, a post-hoc Schefé test was made to differentiate means (95 % confidence, $p < 0.05$). The results in the graphs in the figures represent the mean from «n» independent experiments (n = number of patients) ± standard error of the mean (SEM) or as representative results, as indicated. The GraphPad PRISM version 5 statistical software package (La Jolla, CA, USA) was used to perform one-way analysis of variance followed by Bonferroni's post-hoc comparisons test. Statistically significant differences between experimental conditions were determined by paired comparison test. $P \leq 0.05$ was considered statistically significant.

Table 1. Characterization of commercial fucoidans from Fucus vesiculosus, Macrocystis pyrifera and Undaria pinnatifida brown seaweeds

	Fucoidans from		
	Fucus vesiculosus	***Macrocystis pyrifera***	***Undaria pinnatifida***
Oligosaccharides (%)			
O-Fucose	43.45 ± 0.04^{a}	27.07 ± 0.70^{b}	27.10 ± 0.32^{b}
O-Galactose	6.26 ± 0.07^{b}	6.70 ± 0.67^{b}	24.78 ± 0.69^{a}
O-Glucose	1.77 ± 0.33^{a}	2.22 ± 0.16^{a}	–
Formic acid	6.92 ± 0.57	–	–
Acetyl groups	3.13 ± 0.42	–	–
Sulfate content (mg sulfate/g fucoidan)	353.85 ± 2.36^{b}	338.84 ± 4.72^{c}	384.44 ± 1.93^{a}
Phloroglucinol content (mg phloroglucinol/g fucoidan)	25.39 ± 0.21^{a}	10.54 ± 0.18^{b}	4.26 ± 0.04^{c}
TEAC value (mg trolox/g fucoidan)	65.60 ± 2.09^{a}	14.39 ± 0.58^{b}	5.36 ± 2.09^{c}

Data are presented as mean ± standard deviation. Data values in a row with different superscript letters are significantly different at the $p \leq 0.05$ level.

3. Results and discussion

3.1. Characterization of fucoidans

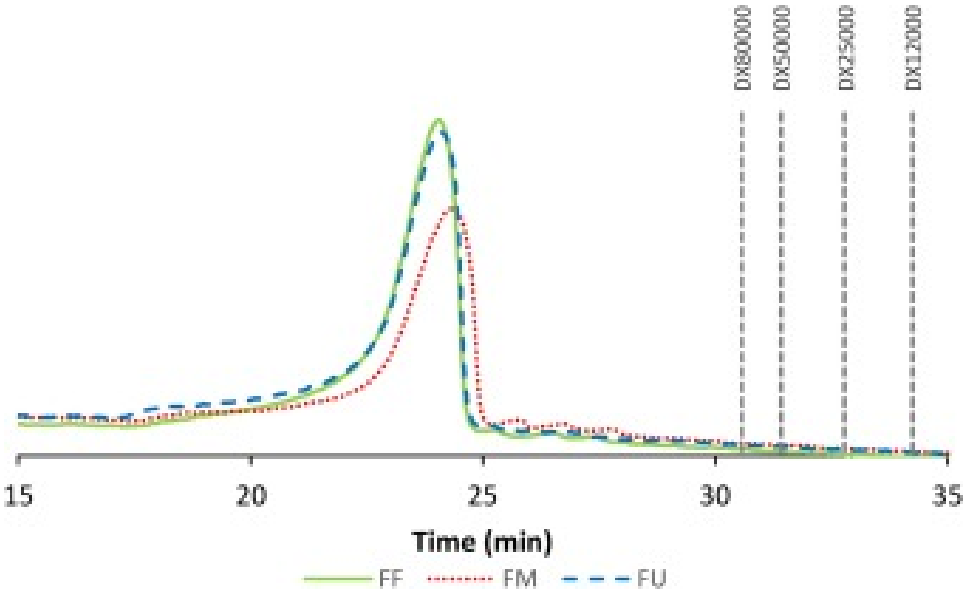
Characterization of commercial fucoidans from F. vesiculosus, M. pyrifera and U. pinnatifida is summarized in Table 1, the composition of the fucoidans is closely associated with the biological activities. In all cases, the fucoidans tested were comprised mainly of fucose, followed by galactose and glucose. Also, formic acid and acetyl groups were found for fucoidan from F. vesiculosus. Other authors showed fucose as the main saccharide in commercial fucoidan from F. vesiculosus and in the fucoidan extracted from Sargassum sp. (Ale, Mikkelsen et al., 2011; Lu et al., 2018). Nevertheless, the maximum fucose content is found in the fucoidan from F. vesiculosus. However, according the literature, fucoidan from U. pinnatifida is comprised mainly of galactose and fucose (Koh et al., 2019), this premise is in consistency with the present work. Other important compound related with the structure and properties of fucoidans is the sulfate content.

The results were similar in all samples, but the maximum was obtained for U. pinnatifida fucoidan. Both the phlorotannin and TEAC value were higher for the sample from Fucus vesiculosus; besides, the fucose/sulfate ratio was maximum also for this fucoidan. These results were in coherence with other authors, the high phlorotannin content contributes to the antioxidant activity, although the sulfate/fucose content ratio could also be a sign of this activity (Wang, Zhang, Zhang, & Li, 2008). The results of fucose and sulfate content related with the antioxidant activity could suggest that the fucose content could be related to the antioxidant activity being maximum in both for F. vesiculosus. Nevertheless, despite the fucoidan structure could influences its biological activity, in some models no influence of the structural features has been observed. For instance, in a rat inflammation model, the content of fucose and sulfate and the structural features did not affect the efficacy of fucoidans (Cumashi et al., 2007). Additionally, no protein was detected in the analyses, fact that could be due to purification steps after the extraction from the seaweeds.

3.2. Molar mass distribution

The Fig. 2 exhibited the molar mass distribution of the three commercial fucoidans tested. The spectra show a similar distribution for the fucoidans extracted from M. pyrifera and U. pinnatifida. In all cases the molar mass was greater than 80 kDa, corresponding to the highest standard used. Several works have stated that the molar mass of fucoidan influences the availability and biological activities, and an optimal range needs to be established depending on the final application (Kopplin et al., 2018; Yan, Lin, & Hwang, 2019). Higher molecular weight fucoidans could present lower bioavailability and activity, and also higher toxicity in cells was reported with increasing molecular weights from low (LMWF: 10–50 kDa) to medium (MMWF: 50–100 kDa) and high (HMWF: >100 kDa) (Gupta et al., 2020). Likewise, pro-inflammatory signaling could also be differently modulated from LMWF and HMWF (Park et al., 2010; Shu et al., 2015).

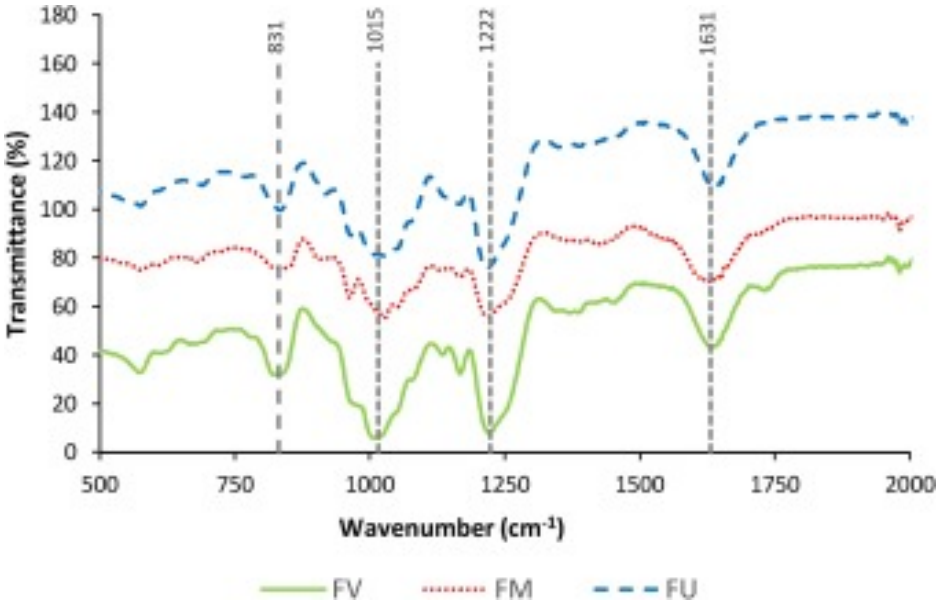
Fig. 2. Molar mass distribution of the commercial fucoidans from the brown seaweeds Fucus vesiculosus (FF), Macrocystis pyrifera (FM) and Undaria pinnatifa (FU). Note here that DX refers to dextran being the following number the molecular weight in Da.



3.3. Fourier-transform infrared spectroscopy (FTIR)

The spectra represented in Fig. 3 collected the FTIR bands from the three commercial fucoidans obtained from Fucus vesiculosus, Macrocystis pyrifera and Undaria pinnatifida. Four signals were observed around 831, 1015, 1222 and 1631 cm−1. The signal represented at 831 cm−1 was associated to bending vibration of Csingle bondOsingle bondS (Zhang et al., 2008). The next signal, obtained at 1015 cm−1 was attributed to Csingle bondO and Csingle bondC stretching vibrations of pyranose ring, regular to the polysaccharides; the band at 1222 cm−1 was related to the asymmetric vibration of sulfate ester group (Sdouble bondO), and at 1631 cm−1 the peak was related with the asymmetric vibrations of elongation of the carboxylate anion (COO-) of pyranose rings (Gómez-Ordóñez & Rupérez, 2011).

Fig. 3. Fourier-transform infrared spectroscopy (FTIR) profiles representation. FTIR profiles representation of the commercial bioactive polymers from different brown seaweed tested F. vesiculosus, M. pyrifera and U. pinnatifida. FF, fucoidan from F. vesiculosus; FM, fucoidan from M. pyrifera; FU, fucoidan from U. pinnatifida.



3.4. Fucoidans inhibit the production of NO induced by IL-1β

In order to evaluate biological actions of fucoidans on articular cells, we first analyzed the effects of different fucoidans on chondrocyte and FLS viability using MMT assays by seeding articular cells with various concentrations of fucoidans (5, 30, 100 μg/mL) in the presence or absence of the pro-inflammatory stimuli IL-1β 5 ng/mL. Our results revealed that no concentration of the different fucoidans showed toxic effect on the articular cells (Fig. 4A and B) as previously studies had detected (Kim & Lee, 2012; Ryu & Chung, 2016; Shu et al., 2015). Then, chondrocytes were stimulated with IL-1β and fucoidans for 48 h and supernatant from the cell culture subjected to the Griess reaction to assess the effects of fucoidans on NO release, a pivotal mediator involved in OA pathogenesis (Amin et al., 2000). As expected (Amin et al., 2000; Wojdasiewicz et al., 2014), IL-1β induced a significant increase in NO production compared to the control group in both cell types (Fig. 4C and D). Previous studies suggested that fucoidans show antioxidant effects through modulation of NO signaling (Park et al., 2017; Phull, Majid, Haq,

Khan, & Kim, 2017). Accordingly, in our study the co-incubation of IL-1β with all fucoidans at 5 μg/mL in the chondrocytes and with only FF 5 μg/mL in synoviocytes resulted in a significant decrease in NO production compared with the IL-1β alone group (Fig. 4C and D). In agreement, different authors observed in vitro that low-molecular weight fucoidans as Fucus vesiculosus inhibit NO release in macrophages and rabbit chondrocytes (Park et al., 2017; Phull et al., 2017). These differences between fucoidans could also reside in the highest content of phlorotannins in FF, well-known antioxidants with recognized NO-scavenging capacity (Koivikko et al., 2005). Conversely, the highest dose of fucoidans (100 ng/mL) did not show any beneficial effect and even enhanced NO release in some cases. We therefore discarded the use of this concentration in all following experiments. Subsequently, the expression of iNOS was evaluated in the articular cells to confirm the previous results. As observed in the Fig. 3E and F, and according to a similar study in keratinocytes (Ryu & Chung, 2016), FF and FU reduced the expression of iNOS induced by IL-1β in chondrocytes. However, and like detected for NO production, only FF was able to slightly attenuate the enzyme levels in FLS.

Fig. 4. Effect of fucoidans on cell viability and NO production induced by IL-1β in articular cells. Osteoarthritic chondrocytes and synoviocytes were incubated for 48 h with fucoidans from Fucus vesiculosus (FF), Macrocystis pyrifera (FM), and Undaria pinnatifida (FU) at 5, 30 and 100 μg/mL w/o, interleukin-1β (IL-1β) (n = 5). Then, cell viability was determined by MTT assay (A and B). NO production (C and D) and inducible nitric oxide synthase (iNOS) expression (E and F) were assayed by griess test and western blot respectively. *, statistically different vs. basal condition, $p < 0.05$; #, statistically different vs. condition stimulated with IL-1β alone, $p < 0.05$.

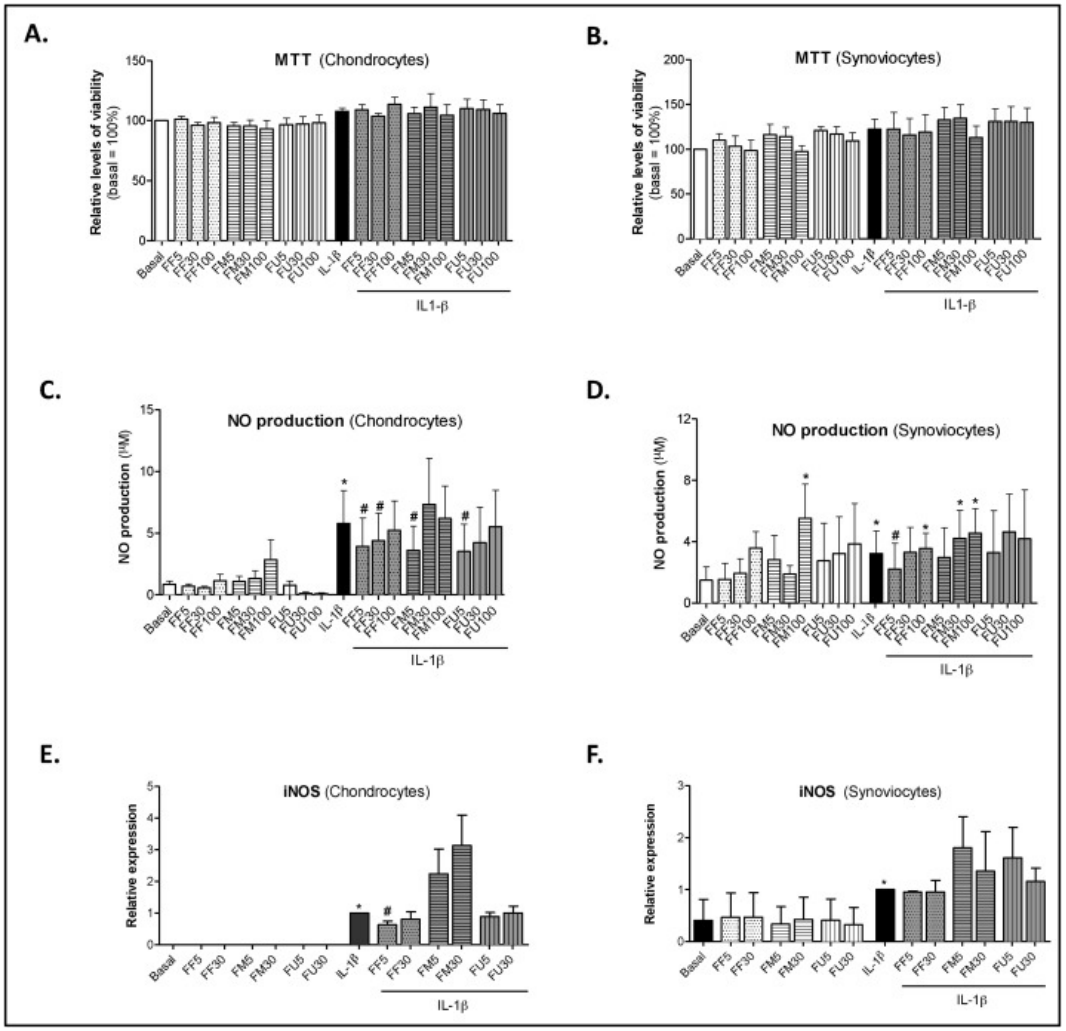
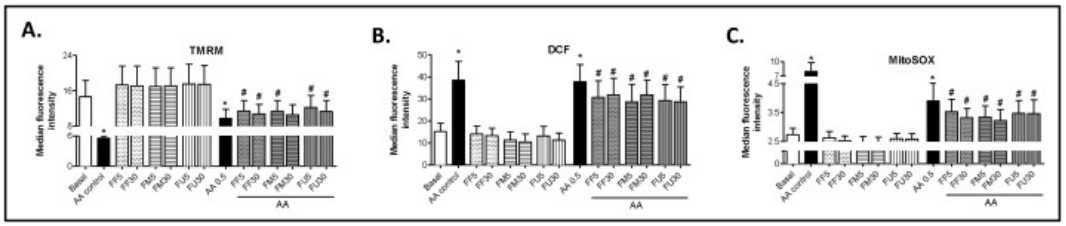


Fig. 5. Effect of fucoidans on mitochondrial dysregulation and associated ROS production. Mitochondrial depolarization and ROS generation were induced in osteoarthritic chondrocytes by Antimicin A (AA; 0.5 μg/mL) in the presence or absence of previously indicated fucoidans at 5 and 30 μg/mL. AA 10 μg/mL were used as positive control of mitochondrial dysregulation. (A) Mitochondrial depolarization was monitorized by TMRM assay (2 h). Production of mitochondrial (B) and cellular ROS (C) were detected by MitoSOX (1 h) and DCE (2 h) fluorescence probes, respectively (n = 5). *, statistically different vs. basal condition, $p < 0.05$; #, statistically different vs. condition incubated with AA alone, $p < 0.05$. FF, fucoidan from Fucus vesiculosus; FM, fucoidan from Macrocystis pyrifera; FU, fucoidan from Undaria pinnatifida; IL-1β, interleukin-1β.



3.5. Fucoidans attenuate mitochondrial impairment and reduce ROS production

Mitochondrial dysfunction is an event taking place in OA chondrocytes that activates pathological pathways in the joint such as inflammation or oxidative stress (Vaamonde-García & López-Armada, 2019). Here, we induced impairment of mitochondrial respiratory chain (MRC) by incubating the chondrocytes with AA 0.5 ng/mL, inhibitor of complex III of MRC. We detected that AA induced a significant loss of membrane potential by TMRM assay. Interestingly, the co-treatment of the cell with all the fucoidans attenuated the mitochondrial depolarization (Fig. 5A). Then, we monitored the production of cytoplasmatic and mitochondrial ROS in the chondrocytes using the fluorescence probes DCFH and MitoSOX™, respectively. As expected, mitochondrial inhibition enhanced the levels of ROS, which were significantly reduced in the presence of the fucoidans (Fig. 5B and C). These results are in accordance with previous publications (Kim et al., 2014; Kim & Lee, 2012). It has been described that polysaccharides with high content of fucose and sulfates, as the three fucoidans here studied, are able to efficiently scavenge free radicals showing antioxidant activities (Kim et al., 2014; Wang et al., 2008).

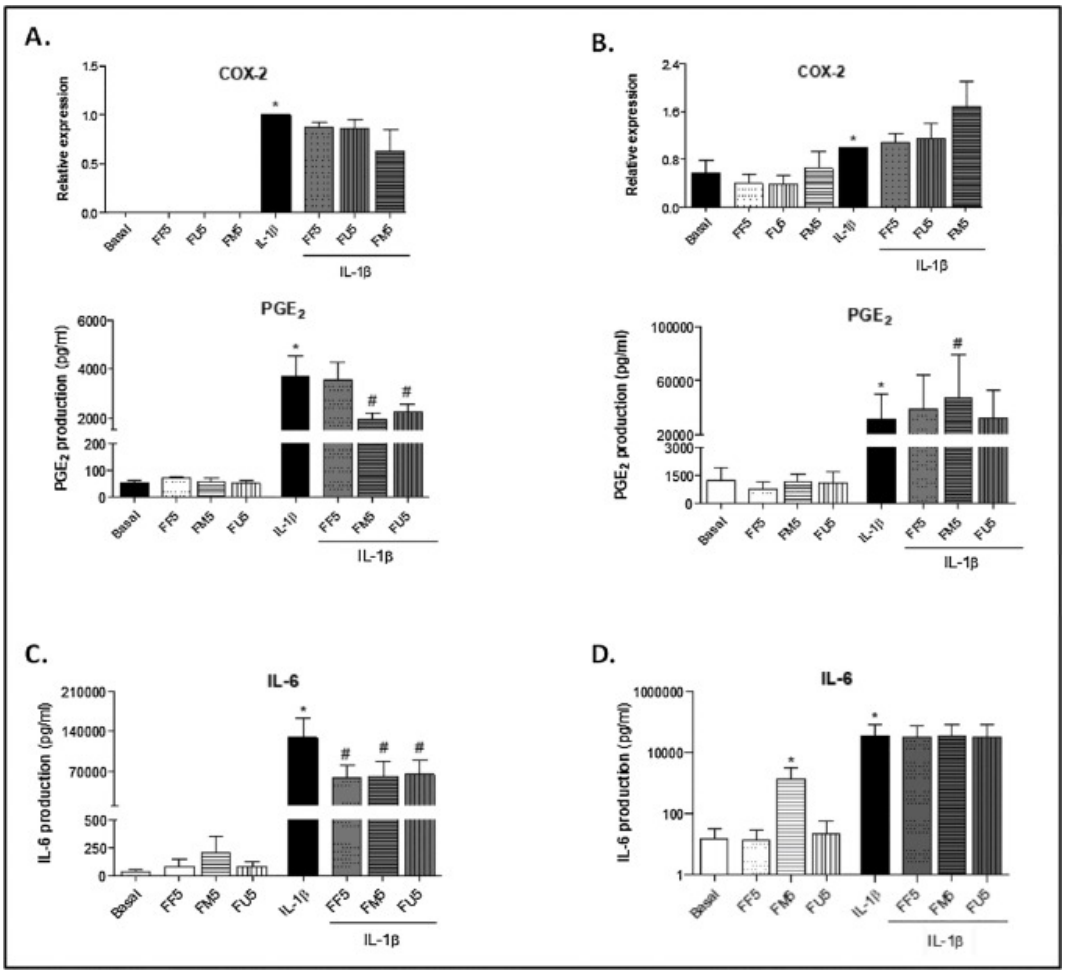
3.6. Fucoidans inhibit the IL-1β -induced expression of pro-inflammatory mediators in chondrocytes

Previous studies described the capacity of fucoidans to modulate the expression of pro-inflammatory mediators like COX-2 (Phull & Kim, 2017a, 2017b; Pozharitskaya, Obluchinskaya, & Shikov, 2020). Thus, chondrocytes and FLS were stimulated with IL-1β in the presence or absence of fucoidans and the protein expression of COX-2 were analyzed by western blot. As shown in the Fig. 6A and B, IL-1β induced a significant increase in the levels of COX-2 in both cell types. The treatment with fucoidans and specially FM reduced the expression of COX-2 in the chondrocytes (Fig. 6A). Conversely, all fucoidans failed to modulate the IL-1β-induced levels of COX-2 in the FLS (Fig. 6B). Accordingly, we observed that the production of PGE2, enzymatic product of COX-2, upregulated by IL-1β was attenuated in the presence of FU and FM in the chondrocytes (Fig. 6C). Whereas, no modulation of PGE2 production was detected in FLS co-treated with all the fucoidans (Fig. 6D). Similarly, the IL-6 release induced by IL-1β in the chondrocytes was significantly inhibited by fucoidans (Fig. 6E), as previous evidence suggested in other cell types (Chen et al., 2017; Li & Ye, 2015) and its use have been recommended as a suitable scaffold material for cartilage tissue engineering applications due to its antioxidant and anti-inflammatory capacities (Sumayya & Muraleedhara Kurup, 2018). However, these modulations were not observed in the FLS (Fig. 6F). In this regard, the inflammatory signal induced by IL-1β in FLS was more patent than in chondrocyte, so that tested concentrations of

fucoidans could hardly attenuate the catabolic effect of the cytokine in these cells. Likewise, it has been described that fucoidans at higher doses or high molecular weight increase apoptosis and induce pro-catabolic phenotype in different cell types like synoviocytes (Park et al., 2010; Shu et al., 2015), discarding its use to specifically control inflammatory signaling in these cells. For instance, Park et al. (2010) reported that in in vitro analyses with macrophages, the HMWF induced the expression of various inflammatory mediators, and enhanced the cellular migration of macrophages but LMWF did not exhibit any pro-inflammatory effects (Park et al., 2010). In addition, as previously indicated, we observed that the highest concentration of these polysaccharides and specially a HMWF like that from Macrocystis pyrifera up-regulated in the FLS the production of catabolic meditator NO (Fig. 4A).

Fucoidans block the IL-1β-induced nuclear translocation of NF-κB and activate Nrf-2/HO-1 signaling in chondrocytes.

Fig. 6. Fucoidan modulation of pro-inflammatory response induced by IL-1β in articular cells. Osteoarthritic chondrocytes (A and C) and synoviocytes (B and D) were stimulated as previously described. Then, COX-2 expression (above) and production of its enzymatic product, PGE2 (below), were measured by western blot and EIA respectively (A and B). Additionally, IL-6 release was assayed by ELISA (C and D) (n = 4). *, statistically different vs. basal condition; #, statistically different vs. condition with IL-1β alone, $p < 0.05$. FF, fucoidan from Fucus vesiculosus; FM, fucoidan from Macrocystis pyrifera; FU, fucoidan from Undaria pinnatifida; IL-1β, interleukin-1β.

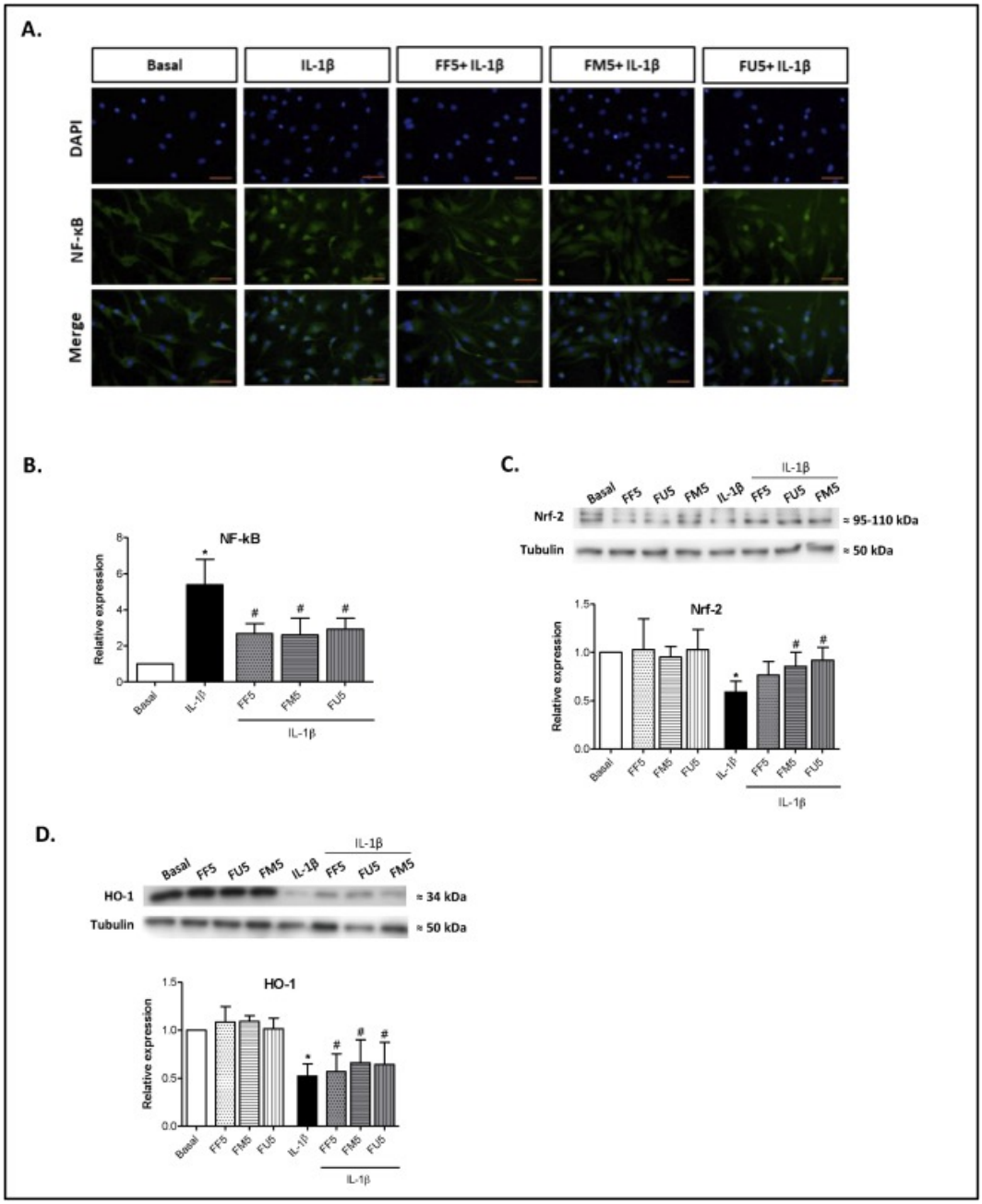


To elucidate the molecular pathways involved in the protective effects of fucoidans in chondrocytes, we analyzed by immunofluorescence the nuclear translocation of NF-κB, indicator of activation of this transcriptional factor, which is known to up-regulate the expression of pro-catabolic pathways in OA (Lepetsos et al., 2019). As shown in Fig. 7A and B, IL-1β significantly increased the nuclear levels of NF-κB, which were diminished in the presence of the all fucoidans. Thus, we hypothesize that fucoidans eject its anti-inflammatory effect in the chondrocytes, at least partially, by reducing the transcriptional activity of NF-κB. Accordingly, it has previously been described in in vitro and in vivo studies that these polysaccharides inhibit NF-κB activation and in turn attenuate the expression of pro-inflammatory mediators (Phull & Kim, 2017a, 2017b; Zhu et al., 2020).

The Nrf-2/HO-1 pathway was also analyzed in our model as different findings have observed that fucoidans could modulate oxidative stress and pro-inflammatory signaling through activation of this anti-oxidant pathway (Ryu & Chung, 2016; Zhu et al., 2020). Chondrocytes stimulated with IL-1β showed by western blot significant lower levels of the biologically relevant Nrf-2 protein (Lau, Tian, Whitman, & Zhang, 2013) (Fig. 7C). Interestingly, the treatment with fucoidans recovery the expression of Nrf-2, achieving significant differences with FU and FM. Likewise, the loss of expression of HO-1, transcriptional target of Nrf-2, induced by IL-1β was significantly attenuated in the presence of all fucoidans. In accordance with modulation of Nrf-2 levels, higher HO expression was observed in cells treated with FU and FM (Fig. 7D). In agreement, a recent study suggested that sulfated polysaccharides show antioxidant potential through the ability to active Nrf-2 signaling pathway (Jayawardena et al., 2020). The differences between fucoidans could reside in the highest ratio sulfate/fucose observed in FU and FM in relation to FV. In relation, it has been described that ratio of sulfate content/fucose may be an effective indicator to antioxidant activity (Wang et al., 2008). Nevertheless, apart from content, the position and substitutions in chemical groups like sulfate could also determine bioactive properties of the fucoidan (Chen et al., 2019).

Taken together, these results suggest that fucoidans could regulate catabolic pathways in chondrocytes through regulation of Nrf-2/HO-1 levels. Similarly, a recent study in keratinocytes observed an attenuation of oxidative stress after induction of HO-1 expression as well as other antioxidant proteins by activation of Nrf-2 pathways (Ryu & Chung, 2016). Thus, the involvement of other Nrf-2 downstream targets in the actions of fucoidans should also be considered and further studies are warranted.

Fig. 7. Effect of fucoidans on IL-1β-induced nuclear translocation of NF-κB and Nrf-2/HO-1 pathway in chondrocytes. Osteoarthritic chondrocytes were stimulated as previously indicated for 1 h. Then, NF-κB translocation was monitorized by inmufluorescence. (A) Representative images showing immunostaining with a NF-κB p65 antibody FITC conjugated (green) (middle panel), counterstain with the nuclear maker DAPI (blue) (upper panel), and merging of both images (bottom panel). (B) Data obtained from performed experiments are represented in the histogram (n = 3). Additionally, the expression of biologically relevant Nrf-2 protein (C) as well as HO-1 (D), one of its most important target genes, were analyzed by western blot. *, statistically different vs. basal condition; #, statistically different vs. condition with IL-1β alone, $p < 0.05$. Fucus vesiculosus; FM, fucoidan from Macrocystis pyrifera; FU, fucoidan from Undaria pinnatifida; IL-1β, interleukin-1β; NF-κB, nuclear factor kappa B; Nrf-2, nuclear factor (erythroid-derived 2)-like 2; HO-1, heme oxygenase-1. Magnification factor 10 × . Scale bar =100 μm.



4. Conclusions and future perspectives

In the present study, we observed that analyzed fucoidans from three different species showed different chemical composition, the maximum phlorotannin content and percentage of fucose was identified in the fucoidan obtained from Fucus vesiculosus, whereas the maximum sulfate content was found in their counterparts extracted from Undaria pinnatifida. In this context, the ratio sulfate:fucose seems critically relevant for fucoidan activity, being the largest ratio for fucoidan from Undaria pinnatifida, followed by Macrocystis pyrifera. However, other factors like concentration and molecular weight of the fucoidan and cell type may influence on their biological effects. Likewise, we detected for the first time to our knowledge that fucoidans show anti-oxidant and anti-inflammatory activities in chondrocytes, as well as protective effects on mitochondrial dysfunction. However, scare effects was found in synoviocytes and even in some cases pro-catabolic actions were detected. The beneficial actions of these polysaccharides could be at least partially mediated by its capacity to activate Nrf-2/HO-1 pathway and to inhibit NF-κB signaling. All together our results shed light on the potential use of fucoidans as natural molecules in the treatment of articular pathologies as OA. Accordingly, recent findings suggest that oral or intraarticular injection of fucoidans promote cartilage regeneration and improve joint damage in different animal models of osteoarthritis (Lu et al., 2019; Sudirman, Ong, Chang, & Kong, 2018). Nevertheless, few clinical trials have been completed until now likely due to lack of comparative studies between different fucoidans and specific experimental models of disease. Thus, our findings along with current studies further encourage the development of future critical trials as well as studies about biological activities that would be interesting to further elucidate how the composition and structure can influence the properties attributed to the fucoidan with interest in the biomedical field.

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CRediT authorship contribution statement

Carlos Vaamonde-García: Conceptualization, Formal analysis, Investigation, Supervision, Writing - review & editing. Noelia Flórez-Fernández: Formal analysis, Investigation, Writing - review & editing. María Dolores Torres: Formal analysis, Investigation, Writing - review & editing. María J. Lamas-Vázquez: Formal analysis, Investigation. Francisco J. Blanco: Writing - review & editing. Herminia Domínguez: Conceptualization, Supervision, Writing - review & editing. Rosa Meijide-Faílde: Conceptualization, Writing - review & editing.

Declaration of Competing Interest

None.

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