

Protective effect of fucoidans from tropical seaweeds against oxidative stress in HepG2 cells

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Abstract Fucoidans are sulfated polysaccharides with proven pharmacological effects localized in the cell wall of marine brown algae. The majority of studies have been performed with temperate brown algal species, but in recent years, the evaluation of species from tropical areas has been growing. The aim of this study was to determine the protective effect of fucoidans extracted from the tropical brown seaweeds *Dictyota ciliolata*, *Padina sanctae-crucis*, and *Sargassum fluitans*, against oxidative stress (OS). The *D. ciliolata* fucoidan (FDc) exhibited the highest reactive oxygen species (ROS) scavenging activity (26%), followed by *P. sanctae-crucis* fucoidan (FPs) (22%) and *S. fluitans* fucoidan (FSf) (14%). No cytotoxic effect was detected for any of the extracted fucoidans at a concentration of 2 mg mL⁻¹. Not only did the fucoidans tested show protective effect against OS by reducing ROS generation, but they also increased the glutathione (GSH) level and restored catalase (CAT) activity. Fucoidans obtained from tropical seaweeds could be used as a potential natural ingredient for functional foods.

Keywords Antioxidant · Fucoidan · Protection · Oxidative stress · Phaeophyceae

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Introduction

Reactive oxygen species (ROS) are highly reactive molecules that are continuously produced within the cell during normal physiological metabolism; they include hydrogen peroxide (H₂O₂), hydroxyl radical (OH·), superoxide anion (O₂⁻), and nitric oxide (NO) (Birben et al. 2012). At low levels, ROS have diverse positive effects on the cell, such as signal transduction and cytotoxicity against pathogens (Dickinson and Chang 2012). However, at high levels, ROS induce damage to various biomolecules such as proteins, lipids, and nucleic acids, which leads to the development of several age-related and chronic diseases such as cancer, diabetes, and neurodegenerative, cardiovascular and liver diseases (Brieger et al. 2012). It has been described that antioxidants can contribute to ameliorate damage induced by oxidative stress (OS), improving the intracellular antioxidant, enzymatic, and non-enzymatic systems (Birben et al. 2012; Brieger et al. 2012). The majority of antioxidants have been isolated from vegetables, fruits, and plants and recently from several marine organisms, particularly seaweeds (Ngo et al. 2011; Freile-Pelegrín and Robledo 2013). Diverse compounds have been isolated from seaweeds with potential antioxidant activity such as fucoxanthins, phlorotannins and fucoidans (Balboa et al. 2013). Fucoidan is a sulfated polysaccharide found exclusively in brown seaweeds (Phaeophyceae). It consists primarily of L-fucose units and sulfate groups, along with small quantities of D-galactose, D-mannose, D-xylose, uronic acids, proteins and phenolic compounds (Li et al. 2008). Extensive research on fucoidans from brown seaweeds has shown many beneficial pharmacological effects in vitro and in vivo, such as anti-inflammatory, antioxidant and hepatoprotective activities (Ananthi et al. 2010; Ross et al., 2012; Vijayavazkar and Vaseela 2012; Xu et al. 2017) and as possible anticancer agents (Lowenthal and Fitton 2015). Clinical studies have also their anticoagulant and antiviral effects (Irhimeh et al. 2009; Negishi et al. 2013), and more

recently, their potential as an adjuvant during chemotherapy in patients with gastric cancer is being used as a nutritional supplement that protects gastric mucosa (Ikeguchi et al. 2015). Due to all of these beneficial effects, fucoidans are considered as a source of bioactive ingredients for functional foods (Vo and Kim 2013).

Many of these studies have been done using fucoidans obtained from temperate brown seaweeds and not until recent years those species from tropical zones (Ananthi et al. 2010; Vasquez et al. 2012; Castro et al. 2015). In this regard, Mexico is considered a country with a vast coast, and in particular at the Caribbean coast of the Yucatan Peninsula where a tropical climate prevails, diverse species of Phaeophyceae, particularly of the order Dictyotales and Fucales, have been described (Robledo 1998). Despite of the above, very few studies have evaluated the pharmacological effect of their fucoidans, with the exception of those reported by Chale-Dzul et al. (2015), who described the hepatoprotective effects of *Turbinaria tricostata* fucoidans. Therefore, the aim of this study was to determine whether fucoidans isolated from *Dictyota ciliolata*, *Padina sanctae-crucis*, and *Sargassum fluitans* have antioxidant properties that can protect against OS induced by different oxidants. We used the HepG2 cell line in vitro as a model to evaluate their protective effects. The partial characterization of the extracted fucoidans was used to discuss the difference between their activities.

Materials and methods

Algal material

Dictyota ciliolata, *Padina sanctae-crucis*, and *Sargassum fluitans* were collected in Puerto Morelos, Mexico (20° 46' 07" N, 86° 57' 14" W) during February 2012. The collected biomass was centrifuged on-site to remove excess seawater using a commercial portable centrifuge and was stored in plastic bags and maintained on ice during transport to the laboratory. The seaweeds were washed thoroughly with freshwater to remove salts, sand, and epiphytes, and were stored at −20 °C. Voucher specimens were identified and deposited at the Mexican National Herbarium (MEXU) Institute of Biology-National Autonomous University of Mexico (IB-UNAM).

Fucoidan extraction

Fucoidans were extracted according to the procedure described by Foley et al. (2011) with modification by Chale-Dzul et al. (2015). Briefly, seaweeds previously stored at −20 °C were thawed and washed with freshwater, and up to 100 g of each seaweed was milled using a commercial blender to obtain a homogeneous paste. The paste was pretreated with 500 mL of ethanol (EtOH) (80% v/v) at room temperature for 12 h. The mixture was filtered, and the solid fraction was

extracted with EtOH (80% v/v) at 70 °C for 12 h. Successive extraction of the solid residue with Milli-Q H₂O was done first at room temperature for 7 h (S1), at 70 °C for 7 h (S2), and at 70 °C for 4 h (S3). The resultant water fractions (a combination of S1, S2, and S3) were treated with 2 M CaCl₂ at room temperature in order to precipitate alginates; after alginate removal by centrifugation at 10,000 rpm for 30 min, the resulting extract was fucoidan-rich. Dialysis was carried out over a 48-h period to decrease salinity employing Milli-Q H₂O with changes every 12 h. Fucoidans obtained were freeze-dried and stored until required.

Chemical analysis

Total carbohydrates and the sulfate content of extracts were quantified utilizing the phenol sulfuric acid method described by Dubois et al. (1956) and the turbidimetric method of Jackson and McCandless (1978), respectively. Uronic acid content was measured with a modification of the carbazole method employing a combination of sulfamate to suppress browning and carbazole to color the uronic acids using D-glucuronic acid as a standard (Blumenkrantz and Asboe-Hanse 1973; Filisetti-Cozzi and Carpita 1991). The protein content was determined according to Bradford (1976) with some modifications. Briefly, 10 mg of each fucoidan was dissolved in 1 mL of deionized water, and then, 10 µL of this solution was placed in a 96-well microplate and mixed with 90 µL of Bradford reagent (Sigma-Aldrich, USA); the plate was incubated at room temperature for 10 min, and finally, the absorbance was measured at 595 nm.

Total phenol content (TPC) was determined using the Folin–Ciocalteu reagent according to Attard (2013) with some modifications. Briefly, 10 mg of each fucoidan was dissolved in 1 mL of deionized water, and then, 10 µL of this solution was mixed with 90 µL of Folin–Ciocalteu reagent (1:10) in a 96-well microplate, followed by the addition of 80 µL of 1 M sodium carbonate (Sigma-Aldrich, USA). The mixture was incubated at room temperature for 20 min in the absence of light, and the absorbance was recorded at 620 nm. The TPC (expressed as % of dry weight) was calculated using a standard curve of phloroglucinol.

The Fourier transform infrared spectroscopy (FTIR) spectra of each one of fucoidans (15 mg) in KB pellets were recorded using the Thermo Nicolet Nexus 670 spectrometer with a DTGS-KBr detector.

Cell culture

The cell lines of human hepatoma (HepG2, ATCC HB-8065) and human embryonic kidney (Hek-293, ATCC-CRL-1573) were purchased from the American Type Culture Collection (ATCC). The cell lines were cultured in sterile Costar 75-cm² flasks containing minimal essential medium (MEN, Gibco)

supplemented with fetal bovine serum (FBS) (10% v/v), 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin for 48 h at 37 °C in an atmosphere of 5% CO₂ and 95% humidity. Cells were subcultured every 5 days by trypsinization with 0.05% trypsin–EDTA solution.

Cytotoxicity assay

The toxicity of *D. ciliolata* fucoidan (FDc), *P. sanctae-crucis* fucoidan (FPs), and *S. fluitans* fucoidan (FSf) was evaluated in HepG2 and Hek-293 cell lines. The cells were plated at a density of 5×10^3 cells well⁻¹ in a flat-bottom, 96-well microtiter plate and were incubated under conditions described in cell culture section. After 48 h of incubation, the cells were treated with appropriate dilutions of FDc, FPs, and FSf (0.125, 0.25, 0.5, 1, and 2 mg mL⁻¹) and incubated for 48 h. At the end of the incubation time, the medium was replaced with fresh new medium and a 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg mL⁻¹ in phosphate-buffered saline (PBS)) and 10 µL of phenazine methosulfate (PMS) solution (5 mg mL⁻¹ in PBS) were added and the plates were incubated at 37 °C for 4 h (Mosmann 1983). After the incubation time, the medium was carefully removed and the formazan crystals generated were solubilized by addition of 100 µL of acidified isopropanol (0.4 N HCl). The plates were read at 540 nm in a microplate reader (GloMax-Multi Detection System with Instinct Software). Dimethyl sulfoxide (DMSO) 0.005% and docetaxel were employed as negative and positive controls, respectively. The concentration of the fucoidans that killed the 50% of the cells (CC₅₀) was calculated using the GraphPad Prism 4.0 software (USA).

Evaluation of the protective effect against H₂O₂-induced OS

Hydrogen peroxide (H₂O₂) can react with transition metals such as Fe²⁺ or Cu⁺; as a result of this, the extremely reactive hydroxyl radical (·OH) is generated, leading to oxidative stress (OS) and cell damage. The HepG2 cell line was plated at a density of 5×10^3 cells well⁻¹ in a flat-bottom, 96-well microtiter plate and was incubated under conditions described in cell culture section. After 48 h of incubation, the cells were treated with the appropriate dilutions of the FDc, FPs, and FSf (0.125, 0.25, 0.5, and 1 mg mL⁻¹) and incubated for 24 h. At the end of the incubation time, hepatic cytotoxicity was induced with 1 mM H₂O₂ for 3 h. Cell survival was determined using the CellTiter-Glo luminescent cell viability assay kit. Each well was treated with a volume of CellTiter-Glo reagent equal to the volume of cell culture (50 µL), mixed for 2 min on an orbital shaker to induce cell lysis, and incubated at room temperature for 10 min, after which the stable luminescent signal was recorded in a microplate reader (GloMax-Multi Detection System with Instinct Software). Non-treated cells, 1 mM H₂O₂ and 1 mM

N-acetyl cysteine (NACT) were used as negative, damage and protective controls, respectively. The viability of the cell was calculated using the GraphPad Prism 4.0 software (USA).

In vitro antioxidant activity

DPPH radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable, free radical compound that is frequently used to measure free radical scavenging capacity of antioxidants. DPPH radical scavenging activity of the fucoidans was determined according to the method of Sharma and Bhat (2009) with modifications described by Chale-Dzul et al. (2015). Briefly, 100 µL of fucoidans at various concentrations (0.125, 0.25, 0.5, 1, and 2 mg mL⁻¹) was mixed with 100 µL of a fresh DPPH solution (300 µM) and then was gently mixed by pipetting and incubated at room temperature for 30 min in the dark. The absorbance recorded at 517 nm ascorbic acid (AA) was used as positive control.

The radical scavenging activity (in percentage) was measured as the decrease in the absorbance of DPPH and was calculated by using the following equation:

Scavenging effect (%)

$$= 1 - \left[\frac{A_{\text{Sample}} - A_{\text{Sample blank}}}{A_{\text{Control}}} \right] \times 100$$

where A_{Control} is the absorbance of the control (DPPH solution without sample), A_{Sample} is the absorbance of the test sample (DPPH solution + test sample) and $A_{\text{Sample blank}}$ is the absorbance of the sample only (sample without DPPH solution).

Measurement of ROS inhibition in HepG2 cells

ROS, including free radicals such as the hydroxyl radical and the superoxide anion, not only play an important role in the cell, but can also induce damage in cells and can lead to the generation of various diseases (Birben et al. 2012). The non-fluorescent probe 5-(and 6) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) diffuses into the cell, where its acetate groups are cleaved by intracellular esterases to non-fluorescent H₂DCF, and this is oxidized in the presence of ROS in dichlorofluorescein (DCF); thus, quantification of DCF can be used as an indication of ROS levels. The effect of fucoidans on ROS inhibition was evaluated in HepG2 cells. The cells were seeded onto 96-well plates (5×10^3 cells well⁻¹) at 37 °C for 48 h. When the cells reached 80–90% confluence, the medium was replaced and the cells were preincubated with CM-H₂DCFDA 1 mM for 30 min at 37 °C in the absence of the light; thereafter, the cells were treated with the fucoidans (0.125, 0.25, 0.5, and 1 mg mL⁻¹) in the presence of 100 mM H₂O₂ for 30 min. Fluorescence was measured at 492/495-nm excitation and 517/527-nm emission in a multi-detection reader (GloMax-

Multi + Detection System with Instinct Software). Non-treated cells, 100 mM H₂O₂, and 1 mM NACT-treated cells were employed as negative, damage and protection controls, respectively.

Determination of GSH levels and CAT activity in HepG2 cells

Glutathione (GSH) is a tripeptide consisting of glutamine, cysteine, and glycine, and is a key component of the non-enzymatic antioxidant cell defense against OS. Catalase (CAT) is a homotetramer belonging to the group of monofunctional CATs. GSH functions directly by scavenging ROS or acting as a substrate for glutathione peroxidase (GPx) and glutathione S-transferase (GST) (Zhang and Forman 2012), whereas CAT removes H₂O₂, when at high concentrations, forming H₂O and O₂, and at low H₂O₂ concentration, CAT acts periodically by oxidizing its substrate (Popovic et al. 2008). The HepG2 cell line was seeded in a 25-cm² flask for 72 h at 37 °C; when cells reached 80–90% confluence, the medium was replaced, and thereafter, the cells were treated with the fucoidans (0.5 and 1 mg mL⁻¹) for 24 h at 37 °C. Cell damage was induced with 400 mM of EtOH for 24 h at 37 °C. After that, the cells were suspended in PBS, washed twice, sonicated, and centrifuged at 10,000×g for 15 min. The supernatant obtained was divided in three aliquots: one used for the determination of the protein content (Bradford assay), the second one for the quantification of GSH levels (glutathione assay kit, Sigma-Aldrich CS0260); and the third aliquot to measure CAT activity (CAT assay kit, Sigma-Aldrich CAT100). Cells without EtOH treatment were negative control, and cells treated with EtOH and EtOH + silymarin were damage and standard control of protection, respectively.

Statistical analysis

All the fucoidan concentrations were evaluated in duplicate at each experiment, and the experiments were performed in triplicate; the results are expressed as mean ± standard deviations (SD). One-way analysis of variance (ANOVA) was used to assess significant differences among treated groups followed by the Dunnett test. Statistical analyses were performed using the GraphPad Prism ver. 4.0 software (USA) to establish statistical significance; values of $p < 0.05$ were considered significant in all cases.

Results

Chemical analysis and fucoidan composition

The chemical composition and fucoidan yield of the tropical brown seaweeds are shown in Table 1. *Dictyota ciliolata* and *P. sanctae-crucis* fucoidans showed a higher content of neutral carbohydrates (65 and 56.8%, respectively), sulfate ester

groups (5.4 and 5.1%, respectively) and uronic acids (13.9 and 11.8%, respectively), when compared to *S. fluitans* fucoidan (50.5, 3.7, and 6.5%), whereas protein content was higher in *S. fluitans* 7.3% (*D. ciliolata* 5.3% and *P. sanctae-crucis* 5.1%). Total phenolic content was higher in *D. ciliolata* fucoidan (2%) and *S. fluitans* fucoidan (1.8%) when compared to that of *P. sanctae-crucis* fucoidan (1.2%).

The FTIR spectra of the fucoidan extracted from *D. ciliolata*, *S. fluitans* and *P. sanctae-crucis* were determined and compared to fucoidan standard from *Fucus vesiculosus* (Fig. 1). Two bands in the 3600–200-cm⁻¹ region, characteristic of all polysaccharides, are evident in all samples. A broad band of around 3346 cm⁻¹ assigned to OH, and the H₂O stretching sugar ring vibration, and another located at 2941 cm⁻¹ corresponding to the C-6 of the pyranoid ring of fucose unit are evident (García-Ríos et al. 2012; Manoj et al. 2013). Other signals that were recorded at 1036 cm⁻¹ were assigned to the guluronic and mannuronic acid residue (an acidic polysaccharide). An intense band at 1628 cm⁻¹ was noted and could be related with the uronic acid content (García-Ríos et al. 2012; Manoj et al. 2013), which is in agreement with the values obtained by chemical analysis.

All three fucoidans exhibited a broad band at 1255 cm⁻¹, which is assigned to the sulfate ester groups (S=O) that are characteristic of fucoidans (García-Ríos et al. 2012; Manoj et al. 2013). Two additional bands were also observed: one at 845 cm⁻¹ indicating the presence of S=O groups in the axial position of the C-4 fucopyranose residue and the other at 825 cm⁻¹, assigned to S=O groups located at C-2 and C-3 in the equatorial position (García-Ríos et al. 2012; Manoj et al. 2013), confirming that the majority of sulfate groups are localized in the C-4 of fucose.

Radical scavenging activities and cytotoxicity

DPPH radical scavenging activity of the three fucoidans is illustrated in Fig. 2. All extracted fucoidans showed a concentration-dependent increase pattern of DPPH scavenging activity, with its maximal activity (27, 22 and 14%, respectively) at a concentration of 2 mg mL⁻¹.

Our results also demonstrated that all three fucoidans did not induce cell damage or compromise viability of HepG2 and Hek-293 cells at 2 mg mL⁻¹ (data not shown from the cell viability study employing the MTT assay). Based on our results, 1 mg mL⁻¹ was chosen as the maximum dose to study the protective effect of fucoidans against OS in a HepG2 cell model of H₂O₂-induced toxicity.

Protective effect of fucoidans on HepG2 cells

Incubation of HepG2 cells with 1 mM H₂O₂ for 3 h (damage control) decreases cell viability by 70% with respect to the untreated cells (negative control), while pretreatment of HepG2 cells for 24 h with NACT reduced the damage induced by

Table 1 Fucoidan yield and chemical composition extracted from *Dictyota ciliolata*, *Padina sanctae-crucis*, and *Sargassum fluitans* (% of dry weight \pm standard deviation)

Fucoidans from:	Yield	Carbohydrates	Sulfate	Uronic acid	Protein	Total phenolic content
<i>Dictyota ciliolata</i>	3.51 \pm 0.95	65.02 \pm 3.19	5.44 \pm 0.85	13.94 \pm 1.17	5.31 \pm 0.14	2.05 \pm 0.18
<i>Padina sanctae-crucis</i>	2.15 \pm 0.85	56.86 \pm 5.28	5.18 \pm 0.41	11.87 \pm 0.64	5.12 \pm 0.38	1.28 \pm 0.05
<i>Sargassum fluitans</i>	2.16 \pm 0.16	50.55 \pm 3.55	3.78 \pm 0.65	6.57 \pm 0.30	7.38 \pm 0.40	1.83 \pm 0.04

H₂O₂, increasing cell viability by 40% more than the damage control (Fig. 3). However, pretreatment of HepG2 cells for 24 h with *D. ciliolata* fucoidan demonstrated a 12% increase on cell viability at 1 mg mL⁻¹, significantly different from that of the damage control. The same was also observed for *S. fluitans* fucoidan at the same concentration, whereas pretreatment of HepG2 cells with *P. sanctae-crucis* fucoidan protected the cells against OS induced by H₂O₂ by increasing 17 and 19% cell viability at 0.5 and 1 mg mL⁻¹, respectively, and significantly different from the damage control.

Effect of fucoidans on intracellular ROS levels in HepG2 cells

The fluorescence spectrometric data (DCF) of ROS inhibition in H₂O₂-treated HepG2 cells are presented in

Fig. 4. HepG2 cells treated with 100 mM H₂O₂ significantly increased intracellular ROS levels by 57% in comparison to non-treated cells (baseline); however, pretreatment with 1 mM NACT reduced ROS levels induced by H₂O₂ by 57% and almost 50% below baseline. Pretreatment with *D. ciliolata* fucoidan at 0.125, 0.25, 0.5, and 1 mg mL⁻¹ significantly decreased ROS generation in relation to the damage control by 17, 29, 57, and 57%, respectively. *P. sanctae-crucis* fucoidan was only active at 0.5 and 1 mg mL⁻¹, with ROS inhibition values of 36 and 46%, respectively. Pretreatment of HepG2 cells with *S. fluitans* fucoidan at 0.25, 0.5 and 1 mg mL⁻¹ completely decreased the damage induced by H₂O₂ and also the baseline, by 21, 29 and 45% respectively. The 1 mg mL⁻¹ dose was similar to the effect shown by the standard NACT. For both *P. sanctae-crucis* and

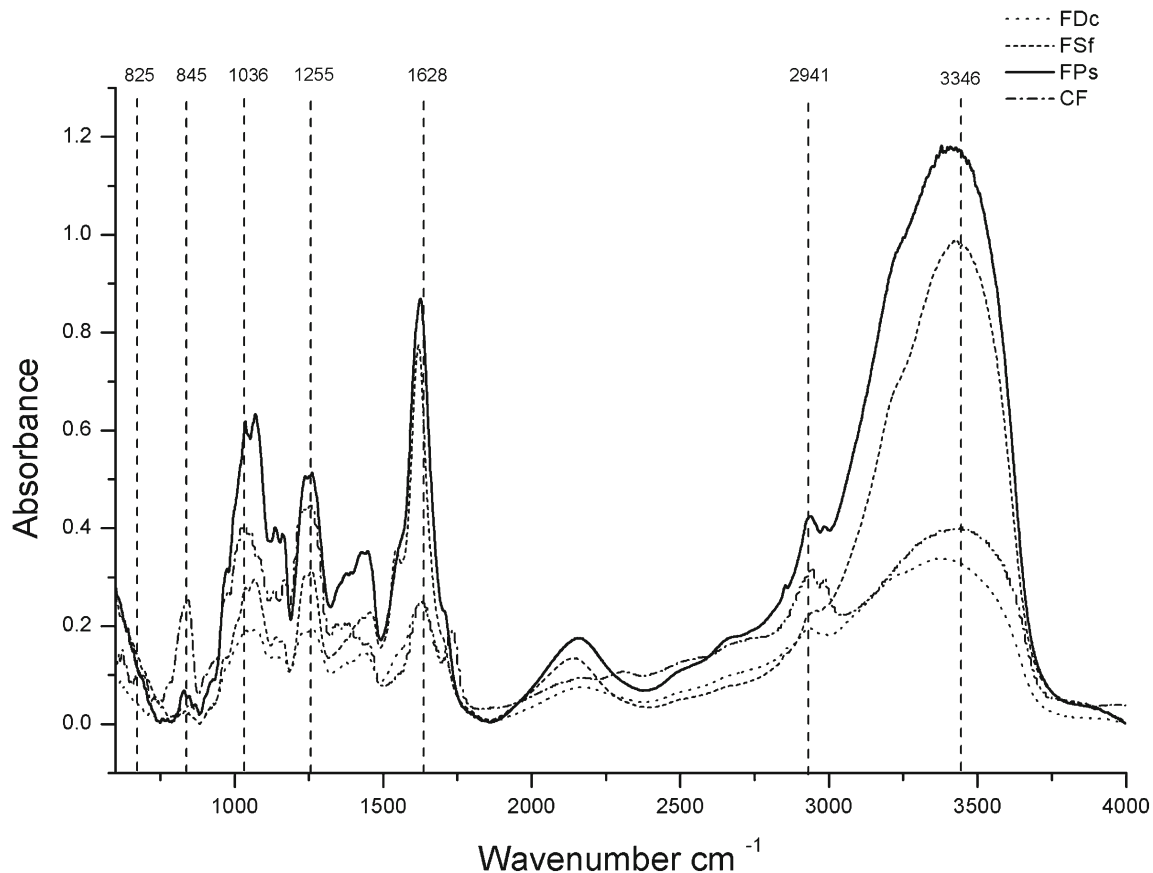
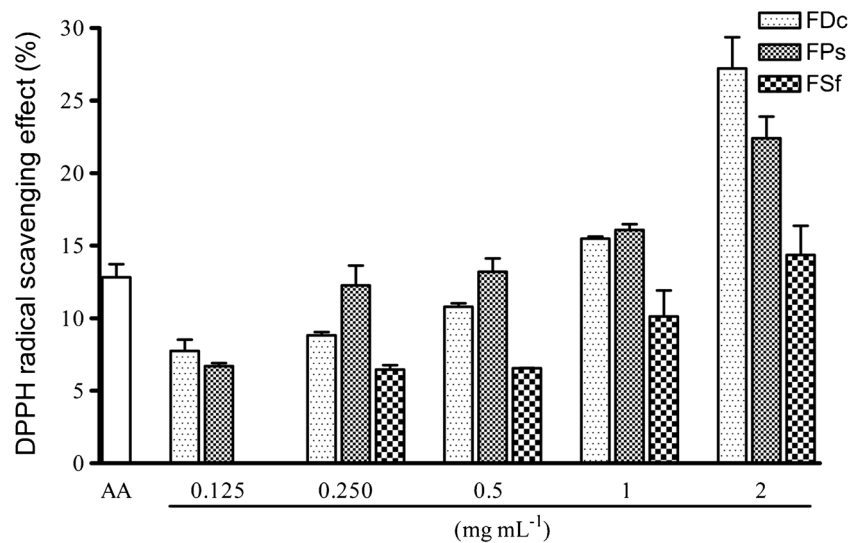
**Fig. 1** Fourier transform infrared spectra (FTIR) of *Dictyota ciliolata* (FDc), *Padina sanctae-crucis* (FPs), and *Sargassum fluitans* fucoidans (FSf). The spectrum of a commercial fucoidan (CF) from *Fucus vesiculosus* is included for comparison

Fig. 2 Effect of *Dictyota ciliolata* (FDc), *Padina sanctae-crucis* (FPs), and *Sargassum fluitans* fucoidans (FSf) on the DPPH scavenging of free radicals in a cell-free system. The amount of the DPPH radical was determined spectrophotometrically at 517 nm. Results are expressed as means \pm standard deviations (SD) ($n = 3$). Ascorbic acid (AA) was used as reference control



S. fluitans fucoidans, the ROS levels were significantly different from the damage control.

Intracellular concentration of reduced glutathione and CAT activity

Treatment of HepG2 cells with 400 mM of EtOH decreased GSH levels by 70% in relation to baseline levels (100%), while pretreatment for 24 h with 0.05 mg mL⁻¹ of silymarin restored the GSH level to that of the baseline in comparison with the damage control (Fig. 5). Pretreatment for 24 h with *D. ciliolata* fucoidan at 0.5 and 1 mg mL⁻¹ restored GSH levels by 15 and 18%, respectively, in comparison with the damage control, whereas pretreatment with *P. sanctae-crucis* fucoidan restored the level of GSH by 30 and 57%,

significantly different from the damage control. *S. fluitans* fucoidan was effective only at 1 mg mL⁻¹, with a GSH restoration by 19% in relation to the damage control. The effect observed can be due to the ability of *S. fluitans* fucoidan to induce GSH synthesis or to their ability to increase or enhance the enzymatic defense system.

In Fig. 6, we present the CAT activity of treated HepG2 cells. The CAT activity was reduced by 35% in relation to baseline (100%) when treated with 400 mM EtOH. As expected, pretreatment with 0.05 mg mL⁻¹ of silymarin avoided the CAT depletion induced by EtOH, reaching a value similar to baseline. Pretreatments with 0.5 and 1 mg mL⁻¹ of *D. ciliolata* fucoidan restored CAT activity by 23 and 35%, respectively, with similar effects exhibited by the silymarin. Pretreatment with *P. sanctae-crucis* fucoidan significantly increased CAT

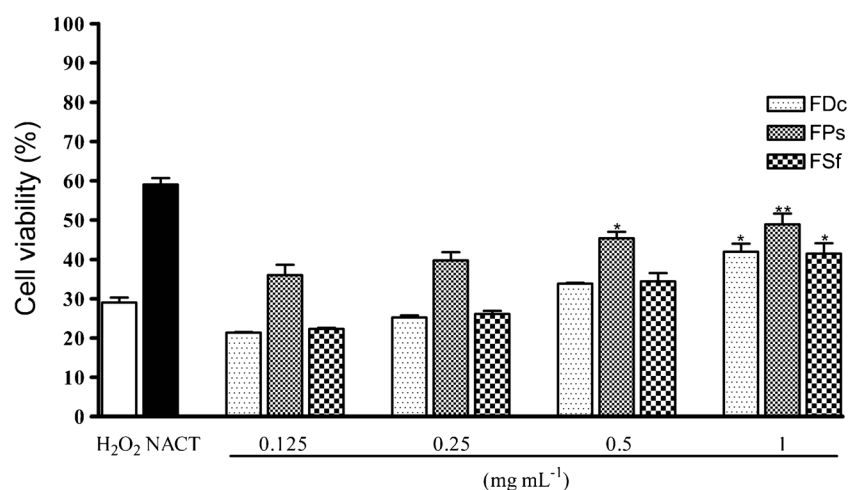


Fig. 3 Protective effect of *Dictyota ciliolata* (FDc), *Padina sanctae-crucis* (FPs), and *Sargassum fluitans* fucoidans (FSf) on H₂O₂-induced damage in HepG2 cells. HepG2 cells were pretreated with four concentrations of FDc, FPs, and FSf, for 24 h; then, damage was induced with 1 mM H₂O₂ for 3 h, and cell viability was determined by ATP assay. Results are expressed as

means \pm standard deviations (SD) ($n = 3$) normalized to the negative control. Cells treated only with 1 mM H₂O₂ were the damage control, and cells treated with 1 mM NACT + 1 mM H₂O₂ were employed as the protection control. One-way analysis of variance (ANOVA) followed by the Dunnett posttest: * $p < 0.05$; ** $p < 0.01$ vs. H₂O₂

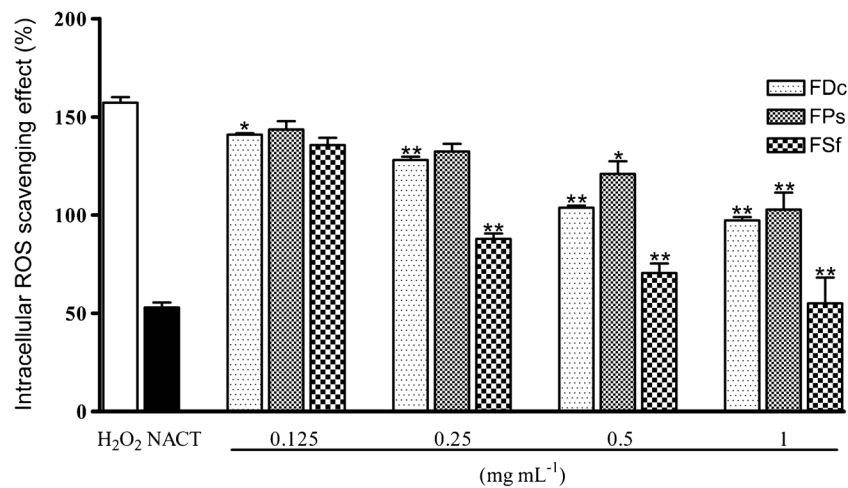


Fig. 4 Effect of *Dictyota ciliolata* (FDc), *Padina sanctae-crucis* (FPs), and *Sargassum fluitans* fucoidans (FSf) on free radical scavenging in a cell system. HepG2 cells were simultaneously treated with four concentrations of FDc, FPs, FSf, and 100 mM H₂O₂ during 30 min. Reactive oxygen species (ROS) generation was detected by fluorescent probe CM-H₂DCFDA. Results are expressed as means ± standard

deviations (SD) ($n = 3$), normalized with the negative control. Cells treated only with 100 mM H₂O₂ were the damage control, and cells treated with 1 mM NACT + 100 mM H₂O₂ were the protection control. One-way analysis of variance (ANOVA) followed by the Dunnett posttest: * $p < 0.05$; ** $p < 0.01$; vs. H₂O₂

activity by 23 and 29% in comparison with the damage control, while in *S. fluitans* fucoidan, the CAT activity was restored to 23 and 29%, respectively, in relation to the damage control.

Discussion

The antioxidant potential of fucoidans is one of the most studied subjects to be used in the prevention or treatment of OS

disorders or associated diseases (Chale-Dzul et al., 2015; Suleira et al. 2016). This activity has been correlated with fucoidan chemical composition. In the present study, we described the chemical composition and the protective effect of fucoidans extracted from the tropical brown seaweeds *D. ciliolata*, *P. sanctae-crucis*, and *S. fluitans*. The chemical composition may influence fucoidan activity and could be related to the ecology of the species studied. In this regard, we found a high content of neutral carbohydrates, sulfate ester groups, and uronic acids, in the fucoidans extracted from

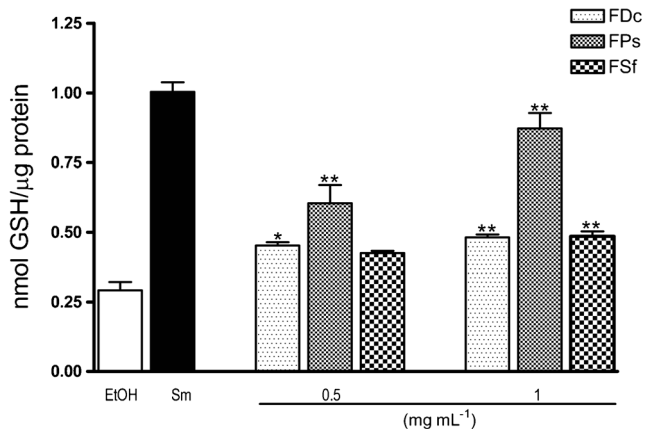


Fig. 5 Effect of *Dictyota ciliolata* (FDc), *Padina sanctae-crucis* (FPs), and *Sargassum fluitans* fucoidans (FSf) on the intracellular level of glutathione (GSH). HepG2 cells were pretreated with two concentrations of FDc, FPs, and FSf for 24 h, and then, damage was induced with 400 mM ethanol (EtOH) for 24 h and the GSH level was determined. Results are expressed as means ± standard deviations (SD) ($n = 3$) normalized to the negative control. Cells treated only with 400 mM EtOH were the damage control, and cells treated with silymarin 0.05 mg mL⁻¹ (Sm) + 400 mM EtOH were the protection control. One-way analysis of variance (ANOVA) followed by the Dunnett posttest: * $p < 0.05$; ** $p < 0.01$; vs. EtOH

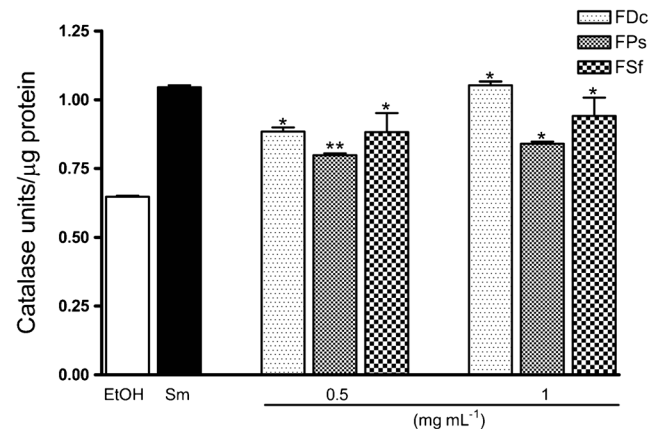


Fig. 6 Effect of *Dictyota ciliolata* (FDc), *Padina sanctae-crucis* (FPs), and *Sargassum fluitans* fucoidans (FSf) on catalase activity (CAT). HepG2 cells were pretreated with two concentrations of FDc, FPs, and FSf for 24 h; then, damage was induced with 400 mM ethanol (EtOH) for 24 h and the GSH level was determined. Results are expressed as means ± standard deviations (SD) ($n = 3$) normalized to the negative control. Cells treated only with 400 mM EtOH were the damage control, and cells treated with silymarin 0.05 mg mL⁻¹ (Sm) + 400 mM EtOH were the protection control. One-way analysis of variance (ANOVA) followed by the Dunnett posttest: * $p < 0.01$; ** $p < 0.05$; vs. EtOH

intertidal species belonging to the order Dictyotales (*D. ciliolata* and *P. sanctae-crucis*), and lower levels in *S. fluitans* (Fucales), which thrives in the sublittoral zone. It has been proposed that seaweeds growing at the intertidal are exposed to higher desiccation and light intensity, environmental stresses that may contribute to higher fucoidan synthesis (Black 1954). Similar chemical compositions from those found in the species studied have been described for fucoidan extract from *Dictyota dichotoma* (Eluvakkal et al. 2010), *Padina boergesenii* (Kordjazi et al. 2013), *Padina gymnospora* (Praveen and Chakaborty 2013), *Sargassum siliquosum* (Vasquez et al. 2012), *Sargassum graminifolium* (Zhang et al. 2012) and *Sargassum wightii* (Marudhupandi and Ajith 2013). FTIR spectra were used to characterize the fucoidans extracted and corroborated the presence of a intense band related with the uronic acid content, a broad band assigned to the sulfate ester groups (S=O) and more interesting two additional bands: one indicating the presence of S=O groups in the axial position of the C-4 fucopyranose residue and a second one assigned to S=O groups located at C-2 and C-3 in the equatorial position (García-Ríos et al. 2012; Manoj et al. 2013). This confirms that the majority of sulfate groups of the fucoidans are localized in the C-4 of fucose; sulfate content and position have been correlated with their antioxidant potential (Marudhupandi et al. 2014).

The major fucoidan impurities described so far which exert low or poor antioxidant activities are proteins (Ananthi et al. 2010; Audibert et al. 2010) and phenolic compounds (Audibert et al. 2010). In this regard, our results showed similar protein contents in *D. ciliolata* fucoidan to that reported for *Dictyota caribaea* (García-Ríos et al. 2012), another tropical species. For *P. sanctae-crucis*, a 1.5-fold more protein was found than those reported in *Padina perindusiata* and *Padina tetrastrumatica* (García-Ríos et al. 2012; Manoj et al. 2013), while in *Sargassum fluitans*, a 2.5-fold more protein was found when compared to *Sargassum tenerrimum* and *Sargassum filipendula* (García-Ríos et al. 2012; Manoj et al. 2013). These differences in protein content have been directly correlated to different factors, including species, collection site and seasonality (Balboa et al. 2013). Phenolic compounds are frequently extracted with fucoidans due their great affinity for carbohydrates and they usually cannot be separated during the extraction procedure. Therefore, its measurement in the fucoidan extract is important since the presence of phenolic compounds may mask or influence fucoidan biological activity. High content of phenolic content in *D. ciliolata*, *S. fluitans* and *P. sanctae-crucis* fucoidans is reported in this study; however, previous studies evaluating fucoidan extracts from *Dictyota* or *Padina* species do not report phenolic contents, while those from *Sargassum muticum* reported values similar to our results for *S. fluitans* (Balboa et al. 2013).

The antioxidant potential of the fucoidans from tropical seaweeds was evaluated using a cell-free system. *D. ciliolata* fucoidan showed a higher DPPH scavenging effect, when compared to *P. sanctae-crucis* and *S. fluitans* fucoidans; this may be attributed to its higher phenolic and sulfate content (Table 1). Costa et al. (2010) described the antioxidant activity of the sulfated polysaccharides isolated from *Dictyota menstrualis* and *Dyctiota mertensii* with maximal hydroxyl radical scavenging of 8.7 and 7.5%, respectively, whereas that from *Dictyota cervicornis* and *Dictyota deliculata* with superoxide radical scavenging of 29 and 32%, respectively, all of them at a concentration of 0.5 mg mL⁻¹. Praveen and Chakaborty (2013) described the antioxidant effect of the fractionated sulfated polysaccharide from *P. tetrastrumatica* and *P. gymnospora* with DPPH scavenging of 77 and 44%, respectively, at a concentration of 1 mg mL⁻¹, while Zhang et al. (2012) reported the antioxidant effect of fucoidans from *S. graminifolium* with a DPPH scavenging of 65% at 1 mg mL⁻¹. A similar pattern (76%) was found for *S. siliquosum* extracts at a concentration of 2.5 mg mL⁻¹ (Vasquez et al. 2012) and for *S. tenerrimum* (64%) at 1 mg mL⁻¹ (Vijayavaskar and Vaseela 2012). On the contrary, Costa et al. (2010) have shown that fucoidans obtained from *S. filipendula* does not possess antioxidant activity. This may be related to the sulfate content, since most of the authors have concluded that the antioxidant effect of fucoidans is due to their amount and position of sulfate ester groups in the molecule.

Although many fucoidans show antioxidant potential, they may also exert cytotoxic effects undesirable to their use as functional foods (Vo and Kim 2013). None of fucoidans extracted from the tropical seaweeds studied showed cytotoxicity on HepG2 and Hek-92 cells. This is important since fucoidans from *D. cervicornis*, *D. deliculata*, *D. menstrualis* and *D. mertensii* have shown cytotoxic effects on cervical carcinoma (HeLa) cell line at 1 mg mL⁻¹ (Costa et al. 2010). Marques et al. (2012) reported the cytotoxic effect of fucoidan from *P. gymnospora* on mouse peritoneal macrophage cell line at 0.5 mg mL⁻¹, whereas *S. schroderi* and *S. filipendula* showed cytotoxicity on cervical carcinoma (HeLa) cell line at 1 mg mL⁻¹ (Costa et al. 2010). Previous studies reported no cytotoxicity of fucoidans isolated from *D. ciliolata*, *S. fluitans* and *P. sanctae-crucis* on hepatocellular carcinoma (HepG2), breast adenocarcinoma (MCF-7), prostate carcinoma (LNCaP), and Hek-293 cell lines at the evaluated maximal concentration of 0.5 mg mL⁻¹, similar to our results (Caamal-Fuentes et al. 2014).

The three fucoidans evaluated in this study also showed a dose-dependent intracellular ROS-scavenging effect. It is noteworthy that *S. fluitans* fucoidan was more effective by diminishing the induced experimental damage, a similar pattern also observed for the *D. ciliolata* fucoidan. Fucoidan fractions from *Sargassum horneri* have shown protective

effects in a cell line model at the maximal concentration of 0.5 mg mL^{-1} (Wen et al. 2014), while no reports were found for fucoidans isolated from *Dictyota* or *Padina* species using similar models. The protection assay against OS in HepG2 cells is widely utilized for screening extracts or drugs with a hepatoprotective potential; thus, our results indicated the enormous potential of fucoidan extracts as potential candidates for hepatoprotective agents (Thabrew et al. 1997), although more experiments may be required to corroborate this hypothesis. Some authors have attributed the protective effect against OS induced by fucoidans to their capacity to directly scavenge free radicals, their ability to chelate ferrous and copper ions, and their capacity to regulate the enzymatic defense system that controls intracellular ROS generation (Hong et al. 2011). Moreover, this has also been associated with the content and position of sulfate groups in the fucoidans (Li et al. 2008; Chattopadhyay et al. 2010; Marudhupandi et al. 2014).

The levels of reduced GSH and CAT activity used as markers in non-enzymatic and enzymatic defense systems help us to determine whether fucoidans are capable of regulating these systems. All the fucoidan extracts evaluated restored the GSH levels, with the most effective effects for the *P. sanctae-crucis* fucoidan. For the CAT activity, a similar pattern was observed, and also, *D. ciliolata* fucoidan reached a restoration similar to that observed for the positive control of protection (silymarin). Popovic et al. (2008) have explained the relation between CAT activity and GSH levels; these authors claim that a reduction of GSH leads to an accumulation of H_2O_2 and consequently, an increase in CAT activity, while Hong et al. (2011) postulated that fucoidans could interact directly at the genic level of transcription and induce an increase in the enzymes, GPX, sodium dismutase (SOD), and CAT, involved in the antioxidant defense system. However, in order to elucidate the mechanism by which fucoidans evaluated in this study induce an increase in CAT activity, more research is needed.

To our knowledge, this is the first study evaluating the protective potential against the OS of fucoidans from the tropical brown seaweeds *D. ciliolata*, *S. fluitans* and *P. sanctae-crucis* on cell lines. From our results, we can conclude that fucoidans obtained from these three species protect HepG2 cells from OS through regulation of the glutathione level and CAT activity. However, further in vivo and clinical studies are necessary to demonstrate their protective effect and to be considered as candidates for the prevention of OS-related disorders.

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