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Hepatoprotective effect of the fucoidan from the brown seaweed *Turbinaria tricostata*

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Abstract Reactive oxygen species (ROS) are involved in initiating and promoting several hepatic diseases. This study was designed to evaluate the in vitro hepatoprotective effect and antioxidative activity of the fucoidan extract from *Turbinaria tricostata* (FTt) from the coast of the Yucatan Peninsula (Mexico). We compared two different mild condition extraction techniques: water vs. salt extraction. The chemical composition and structure of the FTt extracts were determined by Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR). The antioxidant potential was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay. The cytotoxicity was determined in human hepatoma cell (HepG2) and human embryonic kidney cells (Hek-293). The hepatoprotective effect of fucoidan extracts was evaluated by using the hydrogen peroxide (H_2O_2)-induced toxicity on HepG2 cells. In order to assess the possible mechanisms of hepatoprotection of the FTt extracts, ROS intracellular inhibition, glutathione (GSH) level, and catalase (CAT) activity were determined. Our results showed that treatment with FTt extracts displayed significant free radical scavenging action against DPPH and induced a hepatoprotective effect by inhibition of ROS generation. This has been attributed to an increase of catalase activity.

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Introduction

Reactive oxygen species (ROS) are commonly produced by cell metabolism. However, a disturbance of this metabolism, for example induced by viruses, bacteria, and/or hepatotoxic chemicals (i.e., ethanol, acetaminophen), may cause a ROS overproduction, leading to DNA degradation, cell membrane disruption, and reductions of proteins and other molecules. As a result of these, a variety of degenerative processes (i.e., atherosclerosis, neurological disorders, etc.) and several liver pathologies can be generated (Aruoma et al. 2006). Liver damage is especially critical because this organ is a major regulator of metabolite flow and detoxification in the body. Liver comprises three major cell types: hepatocytes, endothelial, and stellate cells. An increase in ROS can damage mitochondria of hepatocytes and induce activation of stellate cells, causing an excessive production of extracellular matrix, resulting in liver fibrosis (Moreira 2007). Efficient antioxidant molecules that scavenge radicals or neutralize ROS may prevent or alleviate liver diseases associated with free radical generation.

The absence of modern reliable hepatoprotective drugs has drawn attention to the hepatoprotective effects of naturally occurring compounds from many terrestrial plants based on their antioxidant activity (Kumar et al. 2011). Bioactive compounds from marine organisms, especially antioxidants from algae, are the ones that have attracted the most interest in recent years (Freile-Pelegrín and Robledo 2013). Marine algae are exposed to a combination of ultraviolet light and environmental stressors that readily leads to the formation of ROS. This is particularly evident for tropical algal species (Freile-Pelegrín and Robledo 2013). Despite their exposure to

harmful ROS, healthy algae lack oxidative damage in their structural components indicating the presence of protective antioxidant defense systems, such as vitamins, pigments, polyphenols, and sulfated polysaccharides.

Fucoidan is a complex sulfated polysaccharide found in the fibrillar cell walls and intercellular space of several types of brown algae (Phaeophyceae). It consists primarily of L-fucose and sulfate groups along with small quantities of D-galactose, D-mannose, D-xylose, uronic acids, and sometimes proteins (Li et al. 2008). In particular, fucoidan from several tropical algal species has been shown to play a vital role in human health due to their numerous pharmacological activities including antiviral, anticoagulant, antioxidant, antiinflammatory, and immunomodulatory effects (Table 1). Fucoidan has also been reported to reduce, through its antioxidative capacity, liver chronic injuries and fibrosis induced by carbon tetrachloride and acetaminophen (Hayashi et al. 2008; Kang et al. 2008; Hong et al. 2011) and to protect hepatic tissues by increasing the content of hepatic growth factor (Fukuta and Nakamura 2008; Hong et al. 2011).

It should be noted that both composition and bioactive properties of fucoidan have been related to factors, such as different algal species, different geographic locations, as well as to extraction methods used. This has been shown by Ale et al. (2011) and Ale and Meyer (2013) in recent comprehensive reviews. Nevertheless, these authors stated that despite intensive research, reliable correlations between bioactivity and structural features of fucoidan still have to be clarified. They also argue that preservation of the structural integrity of the fucoidan molecules during extraction appears to be crucial for maintaining their biological activities since different extraction techniques may lead to extraction of a completely different fucoidan with distinct chemical properties. Fucoidans are usually extracted with acid/base solutions as the solvent, involving long extraction times and high volume of diluents, thus affecting to different degrees the integrity of the fucoidan and, therefore, their biological activities (Yang et al. 2008). More recently, Foley et al. (2011) and others (reviewed on Table 1) have used innovative mild condition extraction procedures, using solely water, with a low consumption of solvent and reduced time, to isolate fucoidans with potential use in pharmacological applications.

Tropical species of marine brown algae remain largely unexploited in the coast of Yucatan Peninsula (Mexico). Particularly, species of the orders Fucales (*Sargassum* and *Turbinaria*) and Dictyotales (*Dictyota* and *Padina*) are present in sufficient amounts for their commercial exploitation (Robledo 1998), and several biological activities (antioxidant, antiproliferative, cytotoxic, and antiprotozoal) of different extracts of these four genera have been reported so far (Zubia et al. 2007; Freile-Pelegrín et al. 2008; León-Deniz et al. 2009; Moo-Puc et al. 2008, 2009; Cantillo-Ciau et al. 2010). In particular, the content and bioactivity (cytotoxic, antiproliferative, and antiprotozoal) of fucoidans from these brown seaweeds have also been determined (García-Ríos et al. 2012; Caamal-Fuentes et al. 2013). However, no hepatoprotective effects have been yet reported or described. Among these species, *Turbinaria tricostata* E. S. Barton is abundantly available in the Caribbean coast of Yucatan throughout the year and, in contrast to other fucoidans from brown seaweeds, those isolated from this genus have been poorly investigated.

As part of our search for compounds with biological activity from tropical marine algae from Yucatán, our study aims to evaluate the in vitro hepatoprotective activity of fucoidan from *T. tricostata* (FTt) comparing two different mild condition extraction techniques.

Materials and methods

T. tricostata (Fucales, Sargassaceae) was collected at the Caribbean coast of the Yucatan Peninsula in Puerto Morelos (20° 46′ 07″ N, 86° 57′ 14″ W) during winter of 2012. The collected biomass was centrifuged on site to remove excess seawater using a commercial portable centrifuge, stored in plastic bags, and kept on ice during transport to the laboratory. The seaweeds were washed thoroughly with freshwater to remove salts, sand, and epiphytes and stored at −20 °C. Voucher specimen was identified according to Wynne (2005) and deposited at the MEXU Herbarium (IB-UNAM).

Preparation of fucoidan extracts

Fucoidan extracts were obtaining using two gentle extraction procedures modified from Foley et al. (2011) and Rioux et al. (2007), hereafter named as P1 (water extraction) and P2 (salt extraction $CaCl_2$), respectively. Briefly, for P1, FTt extract was obtained from fresh algal material (100 g). Seaweeds previously stored at −20 °C were thawed, washed with freshwater, and milled using a commercial blender to obtain a homogeneous paste. The homogeneous 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. Subsequent aqueous extraction of the solid residue involved Milli-Q H_2O 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 fraction (combination of S1, S2, and S3) was treated with 2 M $CaCl_2$ at room temperature in order to precipitate alginates. After alginate removal by centrifugation at 10,000 rpm for 30 min, a remaining extract was obtained. Dialysis was carried out over a 48-h period to decrease salinity using Milli-Q H_2O changes every 12 h. Resultant extracts were freeze-dried and stored until required.

For P2, FTt extract was obtained on dried and milled *T. tricostata* (100 g) and pretreated with 500 mL of EtOH

Table 1 Yield, chemical composition, and biological activities of the fucoidans from tropical algal species extracted under different conditions

Species	Brief description of water extraction methods	Yield (%)	Protein (%)	Carbohydrate (%)	Sulfate (%)	Collecting site	Biological activities	References
Turbinaria ornata (Turner) J. Agardh	Dried and ground seaweed (300 g) depigmented with acetone and extracted with hot water at 90–95 °C for 3–4 h, filtered, concentrated, and precipitated with three volumes of EtOH	10.0	10.6	53.9	–	Coast of Tamil Nadu, India	Antioxidant, antiinflammatory	Ananthi et al. (2010)
Turbinaria ornata (Turner) J. Agardh	Dried and ground seaweed (100 g) mixed with H_2O at 95 °C for 15 min and filtered; the precipitate was treated with EtOH, dialyzed, and lyophilized	–	–	53.1	24.6	Mandapan Coast, India	Antioxidant, anticoagulant	Arivuselvan et al. (2011)
Sargassum hemiphyllum (Turner) J. Agardh	Dried and ground seaweed (100 g) extracted with 5 L H_2O at 100 °C for 20 min; supernatant was lyophilized	9.7	3.9	90.6	51.3	Coast of Penghu County, Taiwan	Antiinflammatory	Hwang et al. (2011)
Sargassum tenerrimum J. Agardh	Dried and ground seaweed (100 g) extracted with H_2O at 90–95 °C for 16 h, filtered, concentrated, precipitated with EtOH, centrifuged, and dried with ethyl ether	11.5	0.9	8.2	6.6	Mandapan Coast, India	Antioxidant	Vijayavazkar and Vaseela (2012)
Sargassum siliquosum J. Agardh	Dried and ground seaweed treated with 80 % EtOH at room temperature for 48 h; residue extracted with boiling H_2O for 4 h, filtered, and lyophilized	3.3	–	–	11.0	Barangay, Calatagan, Batangas, Philippines	Antioxidant, hepatoprotector	Vasquez et al. (2012)
Sargassum wightii Greville ex J. Agardh	Dried and ground seaweed (20 g) treated with EtOH at room temperature for 12 h, washed with C_6H_6O, centrifuged; residue extracted with H_2O at 65 °C for 1 h, centrifuged, precipitated with 1 % $CaCl_2$ at 4 °C for 12 h, EtOH added at 4 °C for 4 h, twice. Supernatant was filtered	4.21	–	52.9	29.3	Mandapan Coast, India	Antibacterial	Marudhupandi and Ajith (2013)
Sargassum graminifolium C. Agardh	Dried and ground seaweed incubated with H_2O at 90 °C for 3 h. The residue extracted twice and concentrated at 80 °C adding EtOH for 12 h, centrifuged and the precipitate washed with EtOH 95 %, ethyl ether and trichloroacetic acid	8.1	–	–	–	South coast of China	Antioxidant	Zhang et al. (2012)
Padina boergesenii Allender & Kraft	Dried and ground seaweed extracted with boiling H_2O and precipitation with $CaCl_2$	4.5	–	–	32.6			
Padina tetrastromatica Hauck		1.0	–	–	19.0	Qeshm Island, Iran	Epidermal regeneration	Kordjazi et al. (2013)
Padina gymnospora (Kützing) Sonder	Dried and ground seaweed (200 g) extracted with H_2O at 80–90 °C for 3–4 h, centrifuged, and lyophilized	9.0	–	33.3	5.7	Mandapan, Gulf of Mannar, India	Antioxidant, antiinflammatory	Krishnankartha and Chakaborty (2013)
Padina tetrastromatica Hauck		12.0	–	33.2	3.6			

(85 % *v*/*v*) with constant mechanical stirring for 24 h at room temperature. The EtOH mixture was filtered and the solid residue used for two additional extractions with EtOH. The solvent was separated from the residual seaweed by vacuum filtration using Whatman number 4 filters. Residual seaweed was treated with 350 mL $CaCl_2$ 2 % (*w*/*v*) and stirred at room temperature for 7 h in order to extract fucoidan and to precipitate alginates. Subsequently, the mixture was filtered and the supernatant obtained was freeze-dried to concentrate the fucoidan. The obtained concentrate was dissolved in Milli-Q H_2O and dialyzed at room temperature for 48 h. Resultant fucoidan was recovered by freeze-drying and stored until required.

Chemical analysis

Total carbohydrates and sulfate content of FTt extracts were quantified using 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 using a combination of sulfamate to suppress browning and carbazole to color the uronic acids using D-glucuronic acid as a standard (Blumenkrantz and Asboe-Hansen 1973; Filisetti-Cozzi and Carpita 1991). The protein content was determined according to Bradford (1976) with some modifications. Briefly, 10 mg of fucoidan extract was dissolved in 1 mL of deionized water, and then, 10 μL of this solution was placed in a well of 96-well microtiter plate; immediately, 90 μL of Bradford reagent (Sigma-Aldrich, USA) was added. Samples were incubated for 10 min at room temperature and absorbance measured at 595 nm.

Total phenol content (TPC) of FTt extracts was determined spectrophotometrically using Folin-Ciocalteu reagent according to the method of Attard (2013) with modifications. Briefly, 10 mg of fucoidan extract was diluted in deionized water (1 mL), and then, 10 μL of this solution was mixed with 90 μL of 1:10 Folin-Ciocalteu reagent (Sigma-Aldrich) followed by the addition 80 μL of 1 M sodium carbonate (Sigma-Aldrich). The mixture was incubated at room temperature for 20 min in dark and the absorbance recorded at 620 nm. The TPC (expressed as % of dry weight) was calculated based on a standard curve of phloroglucinol.

The Fourier transform infrared spectroscopy (FTIR) spectra of the FTt extracts (15 mg) in KBr pellets were recorded using Thermo Nicolet Nexus 670 spectrometer with a DTGS KBr detector. The diffuse reflectance sampling technique was used, and over 64 scans were run for each sample. The ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Varian 600 NMR spectrometer. The FTt extracts were exchanged twice with 99.8 % deuterium oxide (D_2O) with intermediate lyophilization and dissolved at 10 mg mL^{-1} in D_2O. Sodium [3-trimethylsilyl 2,2′,3,3′-2-H4] propionate (TSP-d4) was used as an internal reference for proton chemical shifts.

Cell cultures

The cell lines used were human hepatoma cell (HepG2, ATCC-HB-8065) for the evaluation of hepaprotective effect and human embryonic kidney cells (Hek-293, ATCC-CRL-1573) as a model for healthy cells. The cell lines were cultured in sterile Costar 75-cm^2 flasks containing minimum essential medium (MEM) from Gibco supplemented with fetal bovine serum (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 % CO_2 and 95 % humidity. Cells were subcultured every 5 days by trypsinization with 0.05 % trypsin-EDTA solution.

Toxicity assay

The toxicity of the FTt extracts was evaluated in the cell lines HepG2 and Hek-293. The cell lines were plated at a density of 5×10^3 cells per well in a 96-well flat-bottom microtiter plate and were incubated under conditions described in 'Cell cultures' section. After 48 h of incubation, the cells were treated with appropriate dilution of the fucoidan extract (0.125, 0.25, 0.5, 1, and 2 mg mL^{-1}) and incubated for 24 h. At the end of incubation, the plates were rinsed with phosphate-buffered saline (PBS), and the medium was replaced by fresh new medium. Then, 10 μL of 4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mg mL^{-1} in PBS) and 10 μL of phenazine methosulfate (PMS) solution (5 mg mL^{-1} in PBS) were added to each well and incubated for 4 h at 37 °C (Mosmann 1983). The medium was then removed and the formazan crystals formed were dissolved in 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). Cells treated with 0.005 % dimethyl sulfoxide (DMSO) were used as negative control and docetaxel was employed as positive control. The concentration of the extract that killed 50 % of the cells (CC_{50}) was calculated using GraphPad Prism 4.0 Software (USA).

Evaluation of hepaprotective effect of the FTt extracts

The cell line HepG2 was plated at a density of 5×10^3 cells per well in a 96-well flat-bottom microtiter plate and was incubated under conditions described in "Cell cultures" section. After 48 h of incubation, the cells were treated with the appropriate dilution of the fucoidan extract (0.125, 0.25, 0.5, and 1 mg mL^{-1}) and incubated for 24 h. At the end of incubation time, the hepatic cytotoxicity was induced with 1 mM hydrogen peroxide (H_2O_2) for 3 h. The viability was

determined using CellTiter-Glo Luminescent Cell Viability Assay kit, following the manufacturers' instructions. Briefly, each well was treated with a volume of CellTiter-Glo reagent equal to the volume of cell culture medium present (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 stabile luminescent signal was recorded in a multi-detection reader (GloMax-Multi + Detection System with Instinct Software). Nontreated cells, 1 mM H_2O_2, and 1 mM *N*-acetylcysteine (NACT)-treated cells were used as negative, damage, and protection control, respectively. The viability of the cells was calculated using GraphPad Prism 4.0 Software (Ira et al. 1997).

DPPH radical scavenging activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the FTt extracts was determined according to the method of Sharma and Bhat (2009) with some modification. Briefly, 100 μL of fucoidan extract at various concentrations (0.125, 0.25, 0.5, 1, and 2 mg mL^{-1}) was mixed with 100 mL of a daily-prepared DPPH solution (300 μM). The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark. The absorbance was measured at 517 nm. Ascorbic acid was used as a positive control.

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

$$\text{Scavenging effect}(\%) = 1-\left[1-\left(A_{\text{sample}}-A_{\text{sample blank}}\right)/A_{\text{control}}\right] \times 100$$

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 the $A_{\text{sample blank}}$ is the absorbance of the sample only (sample without DPPH solution).

Assessment of ROS inhibition in HepG2 cells

The effect of the FTt extracts on ROS inhibition was evaluated in HepG2 cells. The cells were seeded onto six-well plates (5×10^3 cells per well) for 48 h at 37 °C. When cells reached 80–90 % confluence, the medium was replaced and the cells were pre-incubated with the fluorescent probe chloromethyl-2′,7′-dichlorofluorescein diacetate (CM-H_2DCFDA) 1 mM for 30 min at 37 °C in darkness. After the incubation, cells were treated with the fucoidan extract (0.125, 0.25, 0.5, and 1 mg mL^{-1}) in the presence of 100 mM H_2O_2 for 30 min (Bak et al. 2012). The 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). Nontreated cells, 100 mM H_2O_2, and 1 mM NACT-treated cells were used as negative, damage, and protection control, respectively.

Assessment of reduced glutathione (GSH) level and catalase (CAT) activity in HepG2 cells

For the determination of GSH, the cell line HepG2 was seeded on a 25-cm^2 flask for 72 h at 37 °C. When cells reached 80–90 % confluence, the medium was replaced and the cells were treated with the fucoidan extract (0.5 and 1 mg mL^{-1}) for 24 h at 37 °C. At the end of incubation, the hepatic cytotoxicity was induced with 400 mM of EtOH for 24 h at 37 °C. After this time, cells were washed twice with PBS, suspended with 5-sulfosalicylic acid 5 %, sonicated, and centrifuged at 10,000×*g* for 10 min and the supernatant was collected. The glutathione level and protein were measured by the glutathione assay kit (Sigma-Aldrich) and Bradford assay, respectively. For the glutathione assay, 10 μL of protein extracts was mixed with 150 μL of reaction mix containing 6 U mL^{-1} of glutathione reductase and 1.5 mg mL^{-1} of 3,3′-dithio-bis (6-nitrobenzoic acid) in 100 mM potassium phosphate buffer (pH 7.0) and incubated at room temperature for 5 min. Then, 50 μL of β-nicotinamide adenine dinucleotide phosphate 0.16 mg mL^{-1} in 100 mM potassium phosphate buffer at pH 7.0 was added. Absorbance was measured at 412 nm every minute for 5 min. The reduced glutathione levels were determined by constructing a curve using reduced glutathione as standard. Cells without ethanol treatment were utilized as negative controls, and cells treated with EtOH and EtOH + silymarin 0.05 mg mL^{-1} were damage and standard control of protection, respectively.

For CAT activity assay, the cell line HepG2 was seeded on 25-cm^2 flask for 72 h at 37 °C. When cells reached 80–90 % confluence, the medium was replaced and the cells were treated with the fucoidan extract (0.5 and 1 mg mL^{-1}) for 24 h at 37 °C. Then, cell damage was induced with 400 mM of EtOH for 24 h at 37 °C. After that, cells were suspended in PBS, washed twice, sonicated, and centrifuged at 10,000×*g* for 15 min. The supernatant was obtained and then the catalase activity and protein were measured using a catalase assay kit (Sigma-Aldrich) and the Bradford assay, respectively. For the catalase activity, 25 μL of 200 mM H_2O_2 and 65 μL of potassium phosphate buffer 50 mM were mixed with 10 μL of the sample and then incubated at room temperature for 5 min, whereupon 900 μL of 15 mM sodium azide solution was added. From this reactant solution, 10 μL was mixed with 1 mL of 0.25 mM 4-aminoantipyrine and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid dissolved in 150 mM potassium phosphate buffer (pH 7.0), then incubated at room temperature for 15 min. The absorbance was measured at 520 nm. The CAT activity was calculated as micromole of H_2O_2

decomposed (min mg^{-1} protein). Cells without ethanol treatment were utilized as negative controls, and cells treated with EtOH and EtOH + silymarin 0.05 mg mL^{-1} were damage and standard control of protection, respectively.

Statistical analysis

All results are expressed as mean±SDs. One-way analysis of variance (ANOVA) was used to assess significant differences among treated groups followed by Dunnett's test, and two-tailed Student's *t* test was used to compared two independent groups (P1 and P2). Statistical analyses were performed using GraphPad Prism 4.0 Software, to establish statistical significance; values of $p<0.05$ were considered significant in all cases.

Results

The extraction conditions of fucoidan extracts were evaluated on its chemical properties by comparing two extractions procedures, P1 and P2, described in the methodological section. Basically, P1 can be considered a water extraction method in comparison to P2 (salt extraction) where $CaCl_2$ was used as the extracting solvent at room temperature.

Yield and chemical composition of the fucoidan extracts obtained by using the two different extraction methods are shown in Table 2. The water extraction procedure (P1) had the highest fucoidan yield, as well as carbohydrate and sulfate content. A high content in uronic acids was obtained, although no significant differences were found between P1 and P2. Protein content and TPC, however, were much lower in the FTt extract obtained by P1.

The FTIR spectra of the FTt extracts obtained by the two extraction methods P1 and P2 as well as that of a commercial fucoidan from *Fucus vesiculosus* (for comparison) are shown in Fig. 1. Although all spectra had similar fingerprint bands, minor differences were observed. Commonly to all polysaccharides, two bands in the 3,600–2,000 cm^{-1} region were evident: a broad band at around 3,346 cm^{-1} assigned to the OH and H_2O stretching vibrations and several smaller bands and shoulders at 2,941–2,981 cm^{-1} assigned to the CH stretching in pyranoid ring, and C-6 groups of fucose units were recorded (Kim et al. 2010). A shoulder in the region 1,542–1,547 cm^{-1}, specific for amide-II (Synytsya et al. 2010), was observed in fucoidan obtained by P2. Bands around 1,032 and 1,043 cm^{-1} were also observed, and they arise from guluronic and mannuronic acid residue, probably due to a residual alginate content. Intense bands at around 1,628 and 1,420 cm^{-1} were also noted and could be related to uronic acid content, which is in accordance with the uronic acids content obtained by the chemical analyses.

An absorption band at around 1,730 cm^{-1} assigned to CO stretching vibration was detected only for *F. vesiculosus* (commercial fucoidan), revealing the presence of some O-acetyl groups, similar to those found in fucoidans from other species of the genus (Bilan et al. 2004). Although several biological activities have been assigned to the acetyl group (Teruya et al. 2009), no pieces of evidence of this peak were found in fucoidans from *T. tricostata* (present study) and or in other studies with *Turbinaria turbinata* (García-Ríos et al. 2012), both species collected from the Caribbean coast of the Yucatan Peninsula.

All samples analyzed exhibited a broad band around 1,220–1,260 cm^{-1}, which has been assigned to be due to the presence of sulfate ester groups (S=O), a characteristic component of fucoidans. An additional sulfate absorption band around 845 cm^{-1} (C–O–S, secondary axial sulfate) might indicate that the sulfate group is located at C-4 of the fucopyranosyl residue, whereas those at around 820–825 cm^{-1} could be associated to low amounts of substitution at the equatorial C-2 and C-3 position (Wang et al. 2010), confirming that most of the sulfate groups in fucoidans are on the C-4 of fucose.

A complex NMR spectra of the FTt extracts obtained by the two extraction methods P1 and P2 are shown in Fig. 2. However, spectra included resonances characteristic of fucoidan, such as signals from ring protons (H-2 to H-5) between δ 3.32 and 4.36 ppm. The presence of intense signals of methyl protons H-6 displaced downfield from δ 1.19 to 1.44 ppm confirmed the presence of fucose residues in the extracts (Karmakar et al. 2009; Chattopadhyay et al. 2010). A number of spin systems (δ 5.24 and 5.44 ppm) attributable to anomeric protons of sulfated α-fucose residues were distinguishable in the spectra. A signal appearing at δ 4.91 ppm can

Table 2 Yield and chemical composition (% of dry weight±standard deviation) of the fucoidan extracts from *Turbinaria tricostata* (FTt) obtained by two procedures: P1 (water extraction) and P2 (salt extraction $CaCl_2$)

Procedure	Yield	Carbohydrates	Protein	Sulfate	Uronic acid	TPC
P1	4.40±0.99*	35.5±2.23*	2.81±0.70*	22.57±0.92*	11.38±0.53	3.13±0.11**
P2	1.50±0.57	30.7±0.29	23.19±2.93	19.5±0.84	11.90±0.96	10.62±0.38

TPC total phenolic content

*$p<0.01$, **$p<0.0001$, P1 vs. P2

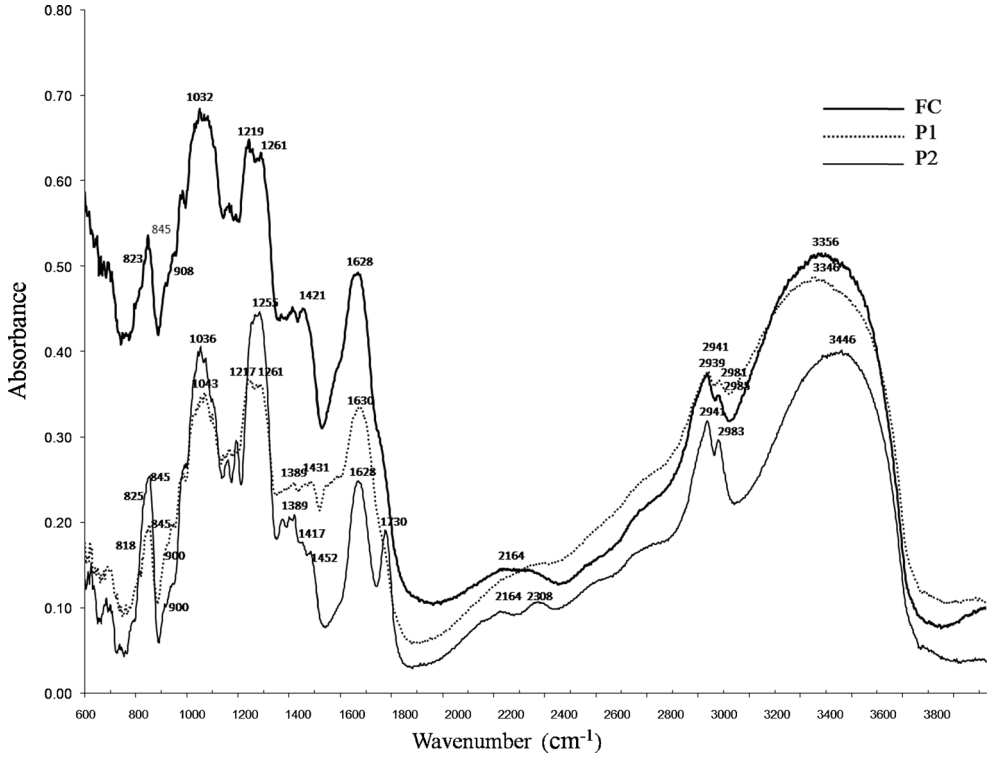

Fig. 1 Fourier transform infrared spectra (*FTIR*) of the fucoidan extracts from *Turbinaria tricostata* (FTt) obtained by two extraction procedures: P1 (water extraction) and P2 (salt extraction $CaCl_2$). Spectrum of a commercial fucoidan (FC) from *Fucus vesiculosus* was included for comparison

be attributed to the H-4 of 4-O-sulfated residues (Bilan et al. 2004) confirming the prediction made by FTIR analysis that sulfate groups are located at position 4 of fucosyl residues. The signals detected between δ 1.82 and 2.70 ppm could be attributed to proportion of proteins (Karmakar et al. 2010) confirming the results obtained by FTIR analysis. Signals between δ 4.45 and 4.90 ppm corresponding to anomeric protons of β-linked sugars were also observed to be possibly related to other xylose and galactose residues (Chattopadhyay et al. 2010).

Scavenging activity of the FTt on DPPH radicals

DPPH is a free radical compound that has been widely used to determine the free radical scavenging ability of antioxidants. As shown in Fig. 3, FTt extracted by P1 and P2 displayed a significant concentration-dependent increase pattern of DPPH scavenging activity. It can be also observed that FTt showed the highest scavenging activity (between 43.2 and 54.6 %) at the highest concentration (2 mg mL^{-1}) with no significant differences between P1 and P2. In the present study, ascorbic acid (extensively used as an antioxidant due to the presence of easily removable hydrogen atoms that effectively donate hydrogen atoms to DPPH radicals) showed a similar scavenging percentage (56.6 %) but at a reduced concentration of 0.012 mg mL^{-1} (data not shown in the graph).

Cytotoxicity and hepatoprotective effect of the FTt on HepG2 cells

Cytotoxic effect is undesirable when the fucoidan will be used as nutraceutical (hepatoprotective). In order to discard any cytotoxic effect, the FTt extracts were tested on HepG2 cell line. Additionally, since any molecule proposed to be used in in vivo studies should be safe in normal cells, the FTt extracts were tested on Hek-293 (a cell line widely used to analyze possible toxic effects on normal cells). FTt extracted by both P1 and P2 did not show any toxicity in hepatocytes (HepG2) and Hek-293 cells at a maximum concentration tested (2 mg mL^{-1}), as shown in Table 3. This indicates that the FTt extracted by both methods are safe when used in healthy organisms, an essential requirement for its possible application as a protective therapeutic agent.

The hepatoprotective effect of the FTt was evaluated on the viability of H_2O_2-treated HepG2 cells. As shown in Fig. 4, induced hepatotoxicity by 1 mM H_2O_2 was confirmed by a reduction of over 70 % of the cell viability (damage control). With the protective control NACT, this reduction was of 40 %. FTt obtained by both extraction methods, P1 and P2, showed a good prevention of cell death at 0.5 and 1 mg mL^{-1}, although at both concentrations, a higher cell viability was observed when compared to damage control group. No significant differences were found in the protective effects of the fucoidan extracted by P1 and P2 at the highest concentrations.

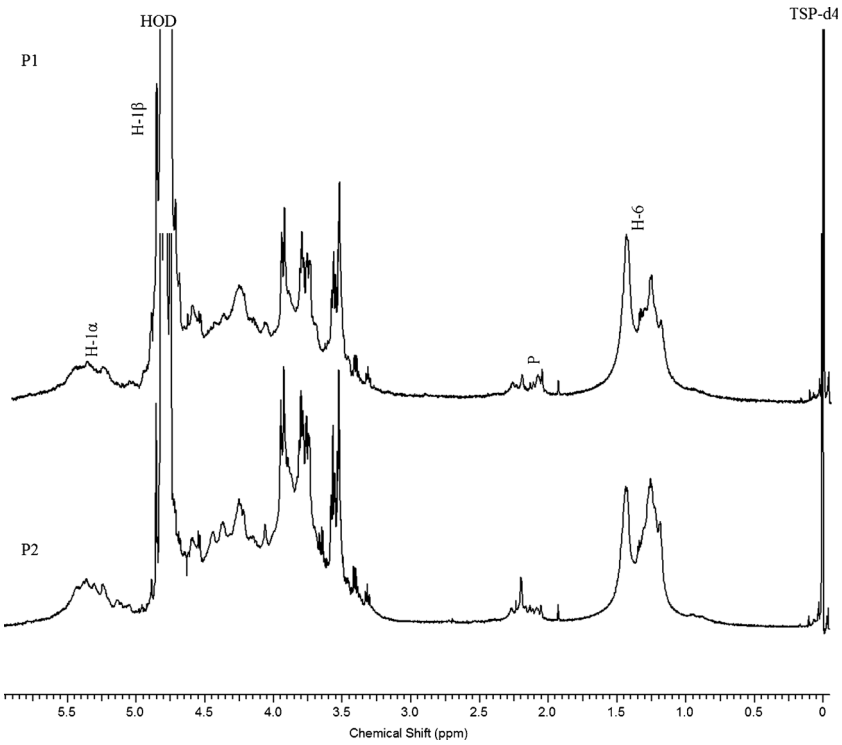

Fig. 2 ^{1}H NMR spectrum at 600 MHz of the fucoidan extracts from *Turbinaria tricostata* obtained by two procedures: P1 (water extraction) and P2 (salt extraction $CaCl_2$). Spectra were recorded at 25 °C for sample in D_2O solution. H1-α, H1-β, and H-6 refer to signals of anomeric protons of α-linked fucose, β-linked sugars, and methyl protons of fucose residues, respectively. The signal for the residual water, proteins, and sodium (3-trimethylsilyl 2,2′, *3,3′-2-H4*) were designated as *HOD*, *P*, and *TSP-d4*, respectively

In vitro antioxidant activity

The hepatoprotective activity of the fucoidans may be attributed to two main factors: its direct antioxidant activity and the mechanism by which they regulate cell antioxidant defense system.

In the present study, a CM-H_2DCFDA staining method was applied to examine whether the FTt was able to inhibit ROS production in H_2O_2-treated HepG2 cells and results are shown in Fig. 5. H_2O_2 is produced endogenously by several physiological processes and has been often used as a model to investigate the mechanisms of cell injury by oxidative stress. When HepG2 cells were treated with 100 mM H_2O_2, ROS significantly increase (over 57 %) when compared to nontreated HepG2 cells (negative control group), and as expected, 1 mM NACT treatment showed a significant decrease of ROS (~66 %) when compared to damage control group. In general, the pretreatment with the FTt extracted by both methods (P1 and P2) showed a decreased dose dependency of formation of ROS, with noticeable lower values at 0.5–1 mg mL^{-1} for the fucoidan extracted by P2 (Fig. 5).

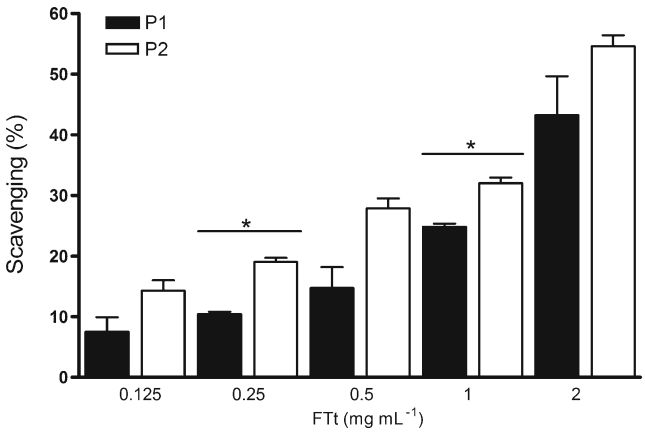

Fig. 3 DPPH radical scavenging activity of the fucoidan extracts from *Turbinaria tricostata* (*FTt*) obtained by two extraction procedures: P1 (water extraction) and P2 (salt extraction $CaCl_2$). Data are expressed as means±standard deviation (n=3). *p<0.05, P1 vs. P2

Enzymatic and nonenzymatic endogenous defense systems are commonly used by cells to protect against ROS. In order to investigate whether the antioxidant properties of the FTt extracts are related to the induction of nonenzymatic and enzymatic defense system, the effect of the FTt (0.5 and

Table 3 Cytotoxicity of crude fucoidan from *Turbinaria tricostata* (CFTt) obtained by two procedures: P1 (water extraction) and P2 ($CaCl_2$ extraction). Docetaxel was included for comparison

Procedure	Cell lines CC_{50} (μg mL^{-1})	
	Hep-G2	Hek-293
P1	>1,000	>1,000
P2	>1,000	>1,000
Docetaxel	0.23	1.10

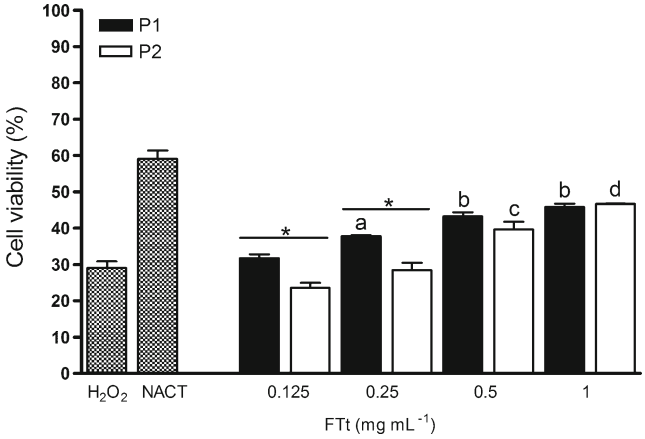

Fig. 4 Hepatoprotective effect of the fucoidan extracts from *Turbinaria tricostata* (*FTt*) obtained by two extraction procedures, P1 (water extraction) and P2 (salt extraction $CaCl_2$), on H_2O_2-induced damage on HepG2 cells. Cells were pretreated with four concentrations of the FTt (0.125, 0.25, 0.5, and 1 mg mL^{-1}) during 24 h after damage was induced with 1 mM H_2O_2 for 3 h (damage control group); the cellular viability was determinate by the ATP assay. Results are expressed as means±standard deviation (n=3), normalized to the negative control. $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.05$, and $^{d}p<0.01$ vs. H_2O_2. *$p<0.01$; P1 vs. P2. Cells treated only with H_2O_2 were the damage control group, and cells treated with 1 mM NACT + H_2O_2 were the protection control group

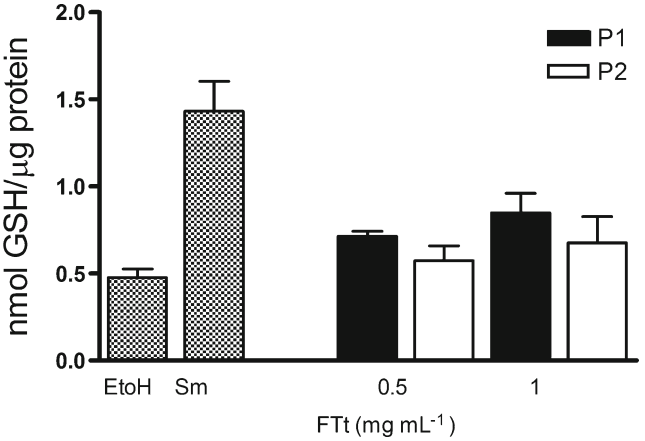

Fig. 6 Intracellular concentration of glutathione (*GSH*) in EtOH-treated HepG2 cells pretreated with the fucoidan extracts of *Turbinaria tricostata* (*FTt*) obtained by two procedures: P1 (water extraction) and P2 (salt extraction $CaCl_2$). Cells were pretreated with two concentrations of the FTt (0.5 and 1 mg mL^{-1}) during 24 h. After EtOH 400 mM for 24 h, damage was induced and glutathione levels were determined. Results are expressed as means±standard deviation (n=3), normalized to the negative control. No significance difference was observed between FTt vs. EtOH and between both extraction methods. Cells treated only with EtOH were the damage control group, and cells treated with silymarin 0.05 mg mL^{-1} (*Sm*) + EtOH were the protection control group

1 mg mL^{-1}) on the intracellular concentration of GHS and CAT activity was measured in EtOH-treated HepG2 cells.

The exposure of HepG2 cells to 400 mM EtOH reduced GSH levels (53 %) in relation to the negative control (referred as 100 % of GSH inhibition), while the pretreatment with silymarin 0.05 mg mL^{-1} avoided GSH inhibition (Fig. 6). Pretreatment with the FTt (0.5 and 1 mg mL^{-1}) extracted by P1 showed a decrease on GSH inhibition by 54 and 30 %, respectively, in relation to damage control group. Pretreatment with the FTt (0.5 and 1 mg mL^{-1}) extracted by P2 also showed a reduced inhibition of GSH levels (81.1 and 62.2 %, respectively) in relation to the damage control group. No significant differences were found between P1 and P2 pretreatment in the GSH inhibition (Fig. 6).

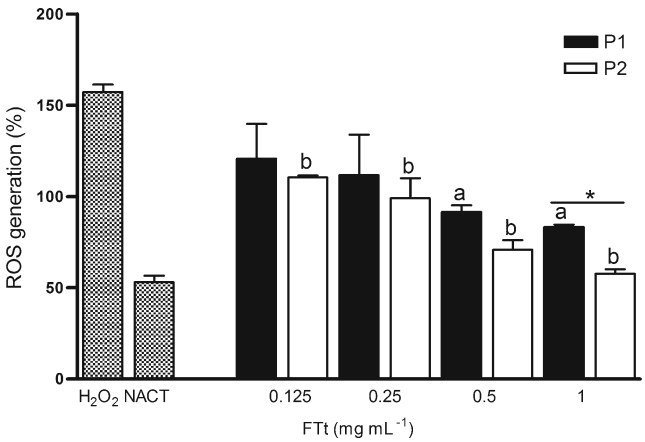

Fig. 5 ROS inhibition of the fucoidan extracts from *Turbinaria tricostata* (*FTt*) obtained by two extraction procedures, P1 (water extraction) and P2 (salt extraction $CaCl_2$), on HepG2 cells treated with H_2O_2. Cells were simultaneously treated with four concentrations of the FTt (0.125, 0.25, 0.5, and 1 mg mL^{-1}) and 100 mM H_2O_2 during 30 min. ROS generation was monitored by fluorescent probe CM-H_2DCFDA. Results are expressed as means±standard deviation (n=3), normalized with the negative control. $^{a}p<0.05$, $^{b}p<0.01$ vs. H_2O_2. *$p<0.01$; P1 vs. P2. Cells treated only with H_2O_2 were the damage control group, and cells treated with 1 mM NACT + H_2O_2 were the protection control group

As shown in Fig. 7, cells treated with 400 mM EtOH showed a reduction of 17 % of the CAT activity in relation to the negative control (considered as 100 % inhibition). As expected, pretreatment with silymarin showed a complete restoration of the CAT activity when compared to damage control group. Pretreatment with the FTt (0.5 and 1 mg mL^{-1}) extracted by P1 showed a complete restoration of CAT activity when compared to damage control group ($p<0.01$) with similar activities to protective control group (silymarin). On the contrary, pretreatment with the FTt extracted by P2 did not show CAT activity restoration. Treatment with the FTt extracted by the two methods showed a different pattern of effect and was significantly different at 0.5 and 1 mg mL^{-1} ($p<0.001$ and $p<0.0001$, respectively).

Discussion

Fucoidans extracted from a tropical brown seaweed were evaluated for its potential in vitro hepatoprotective activity using hepatotoxicity induced by H_2O_2. This is the first study evaluating the hepatoprotective potential of fucoidan from *T. tricostata* from Yucatan in an in vitro model of cell lines,

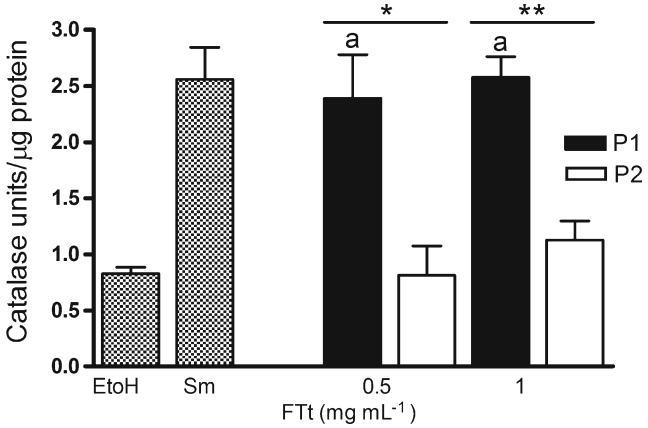

Fig. 7 Catalase (*CAT*) activity in ethanol-treated HepG2 cells with the fucoidan extracts of *Turbinaria tricostata* (*FTt*) obtained by two procedures: P1 (water extraction) and P2 (salt extraction $CaCl_2$). Cells were pretreated with two concentrations of the FTt (0.5 and 1 mg mL^{-1}) during 24 h. After EtOH 400 mM for 24 h, damage was induced and catalase activity was determined. Results are expressed as means±standard deviation (n=3), normalized to the negative control. $^{a}p<0.01$ vs. EtOH. $*p<0.001$; $**p<0.0001$; P1 vs. P2. Cells treated only with EtOH were the damage control group, and cells treated with silymarin 0.05 mg mL^{-1} (*Sm*) + EtOH were the protection control group

and its hepatoprotective capacity is discussed in relation to its antioxidant activity. We compared two fucoidan extracts obtained with different mild condition techniques: water vs. salt extraction.

All chemical composition values obtained using both methods, P1 and P2, were in the range of those reported for the fucoidans also isolated with mild condition extraction procedures from other tropical species (Table 1). In the present study, the water extraction procedure (P1) gave the highest yield, of a fraction that contained the highest carbohydrate and sulfate content. It was noteworthy that P2 yield the highest protein content (over eightfold), in relation to a possible temperature effect (70 °C) on protein during the pretreatment step with ethanol in P1. This results was corroborated by FTIR analysis where, although all FTIR spectra had similar fingerprint bands, minor differences were found, such as a signal for amide-II observed in FTt extract obtained by P2 that could be in relation with the higher protein content obtained by this method (Table 2).

The signals from the FTIR spectra also showed that the most of the sulfate groups in FTt extracted by P1 and P2 were on the C-4 of fucose. This configuration is in agreement with the previous model reported by Patankar et al. (1993) for commercially available fucoidan extracted from *F. vesiculosus*. It is well known that sulfate group position is important for the biological activities of these sulfated polysaccharides. In this regard, the activities related to the concentrations of C-2 sulfate are the common structural feature in anticoagulant fucoidans from *Ascophyllum nodosum* (Chevolot et al. 1999; Chevolot et al. 2001). Silva et al. (2005) also reported that 3-O-sulfation at C-3 of fucose was responsible for the anticoagulant activity of the fucoidan extracted from the tropical brown seaweed *Padina gymnospora*. In relation to fucoidans' antiviral activity, the sulfate located at C-4 of fucopyranosyl units appears to be very important for the anti-herpetic activity of fucoidans from *Cystoseira indica* (Mandal et al. 2007). Based on the FTIR spectra, it could be anticipated that FTt extracted by both procedures (P1 and P2) may be predominantly acidic in nature. Similar results have been reported for the sulfated polysaccharide obtained from *Turbinaria ornata* (Ananthi et al. 2010).

The NMR spectroscopy is a convenient method that produces valuable structural information of polysaccharides. The NMR spectra of FTt extracts obtained by P1 and P2 are complex, similar to those observed for fucoidan from other marine brown seaweeds (Mandal et al., 2007; Karmakar et al. 2009, Karmakar et al. 2010; Chattopadhyay et al. 2010; Ermakova et al. 2011). As preliminary results on the structure of FTt extracts, the presence of various anomeric signals observed in both samples (P1 and P2) confirmed that the fucose residues could have different modifications in their structure such as grade of sulfation, glycosidic linkage positions, and diverse sequence of monosaccharide residues: the anomeric protons of α-fucose residues together with signal of methyl displaced confirmed the presence of sulfated fucose units, while the signals of anomeric protons of β-linked sugar are possible related to xylose and galactose residues. However, further studies to elucidate the complete structure of fucoidan present in *T. tricostata* are required.

FTt extracted by P1 and P2 displayed a significant concentration-dependent increase pattern of scavenging activity. A similar pattern was found for the fucoidan obtained by *T. ornata* (Ananthi et al. 2010) and for a fucoidan fraction from *Turbinaria conoides* from India (Chattopadhyay et al. 2010). The highest scavenging activity (between 43.2 and 54.6 %) obtained at the highest concentration (2 mg mL^{-1}) by P1 and P2 was comparable to that obtained by Chattopadhyay et al. (2010) for a fucoidan of *T. conoides*, where a high scavenging capability on DPPH radicals (61 %) was found at a dosage of 1 mg mL^{-1}. It has been reported that the free radical quenching potential of fucoidan is due to the presence of sulfate groups, one of the most important factors for its biological effects as discussed previously. The antioxidant mechanisms of sulfated polysaccharides are attributed to its strong hydrogen-donating ability, a metal-chelating ability, and its effectiveness as scavengers of free radicals (Wang et al. 2010). However, the relationship between the structure of fucoidan and its antioxidative mechanism has not been yet completed elucidated. It has been reported that a single structural change in the fucoidan molecule (i.e., molecular weight, sulfate and protein content) can result in a considerable difference on its bioactivity (Li et al. 2008; Chattopadhyay et al. 2010; Ale et al. 2011). It is noticeable that FTt extract obtained

by P2 showed a higher DPPH scavenging activity than FTt extract obtained by P1 (regardless of the different contents of sulfate and uronic acids) (Fig. 3) possibly due to other molecules with known antioxidant property, such as phenol compounds (Shibata et al. 2008). Our results showed that the TPC in the FTt extract obtained by P2 was 3.4-fold higher than FTt extract by P1 (Table 2). The higher TPC observed in P2 could be due to extraction method that showed low effectiveness to remove the phenolic compounds unlike of the obtained by P1 (water extraction method) as was also described by Foley et al. (2011).

FTt obtained by both extraction methods, P1 and P2, showed a good prevention of cell death at 0.5 and 1 mg mL^{-1}. This ability of the FTt to protect HepG2 cells against H_2O_2 cytotoxicity provides further evidence for its potential therapeutic usage.

FTt extracts obtained by both methods (P1 and P2) showed a decreased dose dependency of formation of ROS (Fig. 5). These results imply that these FTt extracts may have the ability to directly inhibit ROS production, similar to that reported by Kim et al. (2014) for the fucoidan isolated from *Ecklonia cava* from Korea. It is noteworthy that at the highest doses tested, the most effective inhibition of ROS was observed for the FTt extracted by P2 (salt extraction). The use of $CaCl_2$ in this procedure could be an effective solvent in removing insoluble components, affecting the composition of fucoidan (in particular sulfate content) and, therefore, improving ROS inhibition even though this procedure could yield lower amounts of the fucoidan.

A slight decrease on GSH inhibition in relation to the damage control group was observed when cells were pretreated with the FTt. Glutathione is the main nonenzymatic antioxidant found in cells and represents the first line of defense that scavenge ROS directly or act as a substrate for glutathione peroxidase (GPx) and glutathione *S*-transferase (GST) during the detoxification of ethanol, inducing an decrease of the GSH levels (Li et al. 2004). To our knowledge, this is the first report for in vitro antioxidant potential of fucoidan extracts from *T. tricostata*, although other authors have reported in vivo hepatoprotective effect of different fucoidans (Kang et al. 2008; Hong et al. 2011). When there is not enough GSH to scavenge ROS, other enzymes such as CAT can contribute to regulate ROS. Catalase has two enzymatic pathways depending on the H_2O_2 concentration. If H_2O_2 concentration is high, catalase acts catalytically, removing H_2O_2 by forming H_2O and O_2, whereas at low H_2O_2 concentration and in the presence of a hydrogen donor, catalase acts peroxidically removing H_2O_2 but oxidizing its substrate. Therefore, an increase in the catalase activity could increase antioxidant activity and therefore its hepatoprotective effect (Popovic et al. 2008). From our results, pretreatment with the FTt extracted by P1 showed a complete restoration of catalase activity (despite its low phenolic content) when compared to damage control group with similar activities to protective control group (silymarin). Kang et al. (2008) have reported that fucoidans obtained from the seaweeds *Undaria pinnatifida* and *Saccharina japonica* induced CAT activity restoration in an in vivo model contributing to their hepatoprotective effects. More recently, Meenakshi et al. (2014) have shown that fucoidan extracted from *Turbinaria decurrens* collected from India improved the antioxidant capacity in ethanol administered rats, increasing their level of enzymatic antioxidants (CAT and GPx) in the liver. The high increase in the CAT activity mediated by the FTt extract obtained by P1 method suggests that this fucoidan extract could be responsible for its antioxidant effect (rather than by its phenolic compounds) and consequently for their hepatoprotective effect. The increase in CAT could be due to a direct effect of the FTt regulating the gene expression of this enzyme, but more studies are necessary to confirm this hypothesis.

In summary, fucoidan from *T. tricostata* from Yucatán obtained by water extraction procedure (P1) showed better characteristics for the prevention of hepatic damage when compared to the $CaCl_2$ extraction procedure (P2). This may be related to the stability of the fucoidan molecules, retaining its natural bioactivity when extracted only with water. The fucoidan of this species could be proposed as a supplement in the prevention of hepatic damage. However, further studies using an in vivo model should be performed to confirm these results.

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