Toxicity and Antioxidant Activity in Vitro and in Vivo of Two *Fucus vesiculosus* Extracts

M. C. Zaragozá,[†] D. López,[†] M. P. Sáiz,[†] M. Poquet,[‡] J. Pérez,[‡]
P. Puig-Parellada,[§] F. Màrmol,[§] P. Simonetti,[‖] C. Gardana,[‖] Y. Lerat,[⊥]
P. Burtin,[⊥] C. Inisan,[#] I. Rousseau,[#] M. Besnard,[#] and M. T. Mitjavila*,[†]

Departments of Physiology and Cellular Biology, Faculty of Biology, University of Barcelona, Av.
Diagonal 645, 08028-Barcelona, Spain, Unit of Pharmacology, Faculty of Medicine, University of
Barcelona, Casanova, 143, 08036-Barcelona, Spain, Department of Food Science and Microbiology,
Division of Human Nutrition, University of Milan, Via Celoria 2, 20133-Milan, Italy, Centre d'Étude
et de Valorisation des Algues (CEVA), BP 3, 22610-Pleubian, France, and Diana Naturals,
Phytonutriance, La gare, BP15, 35560-Antrain, France

The consumption of seaweeds has increased in recent years. However, their adverse and beneficial effects have scarcely been studied. Two extracts from the brown seaweed *Fucus vesiculosus* containing 28.8% polyphenols or 18% polyphenols plus 0.0012% fucoxanthin have been obtained and studied to determine their toxicity in mice and rats and also their antioxidant activity. Both extracts were shown to lack any relevant toxic effects in an acute toxicity test following a 4 week daily treatment in rats. The extracts exhibited antioxidant activity in noncellular systems and in activated RAW 264.7 macrophages, as well as in ex vivo assays in plasma and erythrocytes, after the 4 week treatment in rats. Our ex vivo results indicated that compounds from extract 2 may be more easily absorbed and that the antioxidants in their parent or metabolized form are more active. These findings support the view that the daily consumption of *F. vesiculosus* extract 2 (Healsea) would have potential benefits to humans.

INTRODUCTION

The Western diet is rich in polyphenols and carotenoids. Seaweeds, which are widely consumed in Asia, are an important source of such compounds. A variety of in vitro studies have shown that polyphenols and carotenoids exhibit antioxidant activity (*1, 2*). Flavonoids act either by blocking the generation of hypervalent metal forms (*3*), by scavenging free radicals (*4*), or by breaking lipid peroxidation chain reactions (*5*). Carotenoids are also capable of reacting with radical species (*2*). Such antioxidant activities of flavonoids and carotenoids may protect cell constituents against oxidative damage, thereby limiting the risk of diseases associated with oxidative stress and, thus, the inflammatory processes when involved.

An inflammatory response is characterized by the attraction of large amounts of leukocytes (neutrophyles, monocytes-macrophages, and mast cells) to the inflamed area. These inflammatory cells are triggered by mediators of inflammation and generate superoxide anion ($O_2^{\bullet-}$) and nitric oxide (NO) radicals. Thus, antioxidant and anti-inflammatory activities are very close to each other.

Fucus vesiculosus is a brown seaweed species rich in phlorotannins (polyphenols present in brown seaweeds that consist of oligomers or polymers of phloroglucinol or 1,3,5-trihydroxybenzene) (*6*), which also contains fucoxanthin, a major marine carotenoid. *F. vesiculosus* is considered a source of minerals, iodine, proteins, and fiber. However, no toxicological studies on this seaweed or derived extracts have been performed to date, and very few studies have been conducted on the antioxidant activity of phlorotannins (*7*–*9*) and fucoxanthin (*10, 11*) from seaweeds. Thus, we hypothesized that on the basis of polyphenol and carotenoid contents, *F. vesiculosus* extracts might have an antioxidant effect. To test this hypothesis, from an initial screening, we selected two *F. vesiculosus* extracts, one rich in phlorotannins and the other rich in phlorotannins and fucoxanthin, to compare their effects. We then evaluated the acute and 4 week toxicities and the antioxidant properties via three approaches, namely, reducing power, free radical trapping, and enzymatic activities, by several noncellular

* To whom correspondence should be addressed. Tel: +34-93-4021530. Fax: +34-93-4110358. E-mail: mmitjavila@ub.edu.
† Department of Physiology, University of Barcelona.
‡ Department of Cellular Biology, University of Barcelona.
§ Unit of Pharmacology, University of Barcelona.
‖ University of Milan.
⊥ Centre d'Étude et de Valorisation des Algues (CEVA).
Diana Naturals.

7774 *J. Agric. Food Chem.,* Vol. 56, No. 17, 2008

Zaragozá et al.

(chemically generated oxidants), cellular (macrophages), and ex vivo (in plasma and erythrocytes after treating rats with two *F. vesiculosus* extracts or with phloroglucinol) tests. The ex vivo tests allowed us to assess differences resulting from the presence of metabolized forms of the parent antioxidants in blood.

MATERIALS AND METHODS

Apparatus, Reagents, and Biological Material. Centricon cartridges were acquired from Millipore (Bedford, MA). Ceramic membranes were from Sepra (Cesano Maderno, Italy). The high-performance liquid chromatograph (HPLC) Alliance and the diode array detection (DAD) system were from Waters (Milford, MA), and the Quattromicro triple quadrupole mass spectrometer and the Masslinx 4.0 software were from Micromass (Beverly, MA). The Symmetry columns were acquired from Waters, and the Synergy Hydro columns were from Phenomenex (Torrence, CA). Cell counter Celltacα was acquired from Nihon (Kohden, Japan). An Easy Lyte Na/K/Cl analyzer was from Medica (Bedford, MA). The MicroOsmometer was acquired from Advanced Instruments (Norwood, MA). The Iso-NOP sensor was from World Precision Instruments (Saratosa, FL). Fucoxanthin, violaxanthin, asthaxanthin, and echinenone were purchased from DHI (Hoersholm, Denmark). The protein assay was from BioRad (Hercules, CA). Biochemical kits were acquired from Biosystems (Barcelona, Spain), Olympus (Hamburg, Germany), and Randox (Crumlin, United Kingdom). Folin−Ciocalteau reagent, quercetin, phloroglucinol, xanthine (X), xanthine oxidase (XO), nitroblue tetrazolium salts, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, 2,2′-azo-bis-2-amidinopropane (ABAP), luminol, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Cu−Zn superoxide dismutase (Cu−Zn SOD), cytochrome c (horse heart type VI), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), paraoxon, *R*-phycoerythrin, and pyrogallol were purchased from Sigma-Aldrich (St. Louis, MO). Trolox and chloral hydrate were acquired from Fluka Chemie (Buchs, Switzerland). N5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO) was provided by A.G. Scientific, (San Diego, CA). *F. vesiculosus* was obtained from Agrimer (Plougerneau, France) and Centre d'Étude et de Valorisation des Algues (CEVA, Pleubian, France). Swiss mice and Sprague−Dawley rats were provided by Harlan Interfauna Ibérica (Barcelona, Spain). RAW 264.7 macrophages were from American Type Culture Collection (Rockville, MD).

Plant Material. *F. vesiculosus* raw material was obtained at two different sites from the French North Coast. *F. vesiculosus* was harvested in late winter/spring and in late summer, as the highest and similar contents in polyphenolic compounds were found in both areas. Once harvested, the seaweeds were quickly washed with soft water, dried at 100 °C for 1 h, and then finely ground (120−250 μm).

Preparation of the *F. vesiculosus* Extracts. *Extract 1.* A hydroethanolic extraction (30−35% ethanol) of *F. vesiculosus* (10% w/w) was carried out at room temperature under mechanical stirring for 4 h. After filtration on a filter press, the liquid phase underwent an initial purification step to remove the alginates by precipitating them in the presence of excess $CaCl_2$. Following filtration, the liquid phase underwent a second purification step involving diafiltration to remove the iodine and the low molecular weight compounds and was freeze-dried to obtain a powder extract.

Extract 2. A hydroethanolic extraction with high-percent ethanol (50−70%) of *F. vesiculosus* (Healsea) (10% w/w) was conducted to solubilize a higher amount of carotenoids at room temperature under mechanical stirring for 2 h. After filtration on a filter press, the liquid phase underwent an initial purification step to remove the alginates by precipitating them in the presence of excess $CaCl_2$. After solid−liquid separation, a second extraction was performed under the same conditions. The two liquid phases were then blended, submitted to a diafiltration to remove the iodine and the low molecular weight compounds, and freeze-dried to obtain a powder extract.

Characterization of the *F. vesiculosus* Extracts. *Chemical Composition.* The chemical composition of the *F. vesiculosus* extracts was determined by standard methods. Iodine evaluation in seaweed extracts was performed by the method described in the Food Chemical Codex V. The total polyphenols content was evaluated using the colorimetric Folin−Ciocalteau method (*12*), and the molecular weights distribution was performed using Centricon cartridges with cutoffs of 3, 10, 30, and 50 kDa and an ultracentrifugation with ceramic membranes with a cutoff of 300 kDa.

Evaluation of Phloroglucinol and Its Derivatives. Sixty milligrams of each *F. vesiculosus* extract was sonicated in 10 mL of water, and after 10 min, the mixture was filtered, and the resulting solution was adjusted to 20 mL. The mixture was centrifuged at 1000*g* for 2 min, and the supernatant (20 μL) was injected into the HPLC-DAD-MS system. The chromatographic system consisted of an Alliance 2695 equipped with a DAD 2996 and a Quattromicro triple quadrupole mass spectrometer. A 3.5 μm C_{18} Symmetry column 150 mm × 2.0 mm was used for the separation at 0.25 mL/min. The column was maintained at 30 °C, and the separation was performed by means of a linear gradient elution (eluent A, 0.1% HCOOH in water; eluent B, 0.1% HCOOH in acetonitrile). The gradient was as follows: 0% B at time zero, 0−20% B in 20 min, 20−80% B in 5 min, and 80% B for 20 min. Chromatographic data were acquired in the 200−450 nm range and were integrated at 275 nm. The mass spectrometer was operated in negative full-scan mode in the range 100−2000 Da. The capillary voltage was set to 3.0 kV, and the cone voltage was 30 V. The source temperature and the desolvating temperature were 130 and 300 °C, respectively. Data were acquired by using Masslinx 4.0.

Qualitative and Quantitative Evaluation of Carotenoids. One gram of each *F. vesiculosus* extract was sonicated in 70 mL of methanol, and after 30 min, the mixture was filtered, and the resulting solution was adjusted to 100 mL with methanol. The mixture was centrifuged at 1600*g* for 10 min, and the supernatant (10 μL) was injected into the HPLC-DAD-MS system. The chromatographic system was the same as for phloroglucinol evaluation. A 4 μm C_{18} Synergy Hydro column 150 mm × 2.0 mm was used for the separation at 0.3 mL/min. The column was maintained at 30 °C, and the separation was performed by means of a linear gradient elution (eluent A, 2 mM CH_3COONH_4, pH 5.2; eluent B, methanol). The gradient was as follows: 80% B at time zero, 80−100% B in 8 min, and 100% B for 7 min. Chromatographic data were acquired in the 200−700 nm range and were integrated at 445 nm. The mass spectrometer was operated in positive full-scan mode in the range 200−800 Da. The capillary voltage was set to 3.0 kV, and the cone voltage was 20−60 V. The source temperature and the desolvating temperature were 130 and 350 °C, respectively. Data were acquired by using Masslinx 4.0 with the Quan-Optimize option for cone voltage and collision energy optimization.

Antioxidant Activity of the Extracts in Noncellular Systems. Although many techniques can be used to test for antioxidants, there are no approved standard methods. The significance and relevance of natural antioxidants, which are often multifunctional, depend strongly on the test applied. Thus, it is commonly accepted that several tests should be used to correctly assess the capacity of compounds to respond to oxidative stress. We performed some tests simultaneously with the two extracts, with phloroglucinol and when possible with a positive control and a negative control (*13*).

Reducing Power. To evaluate the electron donor capacity (*14*), the seaweed extracts, phloroglucinol and quercetin (positive control), were mixed with 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide, incubated at 50 °C for 20 min, and centrifuged at 2250*g* for 10 min after adding 10% trichloroacetic acid. The upper layer was mixed with 0.1% $FeCl_3$, and the optical density was measured at 690 nm and reported to quercetin activity.

$O_2^{\bullet-}$ Scavenging Activity. This test was based on the reduction of the nitroblue tetrazolium salt to water-soluble formazan by the $O_2^{\bullet-}$ generated by the xanthine/xanthine oxidase (X/XO) system (*15*). The reaction was initiated by adding XO, and we measured the difference in absorbance at 560 nm between 0 and 10 min at 25 °C. The percentage of $O_2^{\bullet-}$ inhibition was then evaluated. The concentration of the sample that inhibited the nitroblue tetrazolium salt reduction by 50% (IC_{50}) was calculated, and lower IC_{50} values pointed to stronger activity.

DPPH Radical Scavenging Activity. DPPH radical is one of the major tests commonly used to evaluate the antioxidant activity of phenolic compounds. The method is based on the reaction of antioxidants with this stable radical in an alcoholic solution (*16*).

Dilutions of *F. vesiculosus* extracts were mixed with 100 μM DPPH solution in ethanol for 30 min at 37 °C. The sample backgrounds were evaluated by adding ascorbic acid (250 μM final concentration). The scavenging potential was compared with a solvent control (0% radical scavenging) and ascorbic acid (100% radical scavenging). The IC_{50} was then calculated.

Total Radical Antioxidant Parameter (TRAP). This test used peroxyl radicals generated by thermal homolysis of ABAP to oxidize antioxidants (*17*) and luminol as an amplifying signal. Trolox, a water-soluble analogue of vitamin E, was used as a positive control. When a stable chemiluminescence signal was obtained, varying concentrations of the antioxidant solution (*F. vesiculosus* extracts or phloroglucinol) were added in a volume of 20 μL. The time required to reach 50% of the initial lecture was registered and reported to trolox activity.

ABTS Radical Cation Scavenging Activity. The ABTS radical cation decolorization test reflected the ability of hydrogen- or electron-donating antioxidants to scavenge this radical (*18*). ABTS radical cation was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. The ABTS radical cation solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 °C. Dilutions (10 μL) of *F. vesiculosus* extracts, ethanol (negative control), or standard solutions of Trolox (positive control) were mixed for 30 s with 1 mL of diluted ABTS radical cation solution, and the absorbance at 734 nm was taken at 30 °C exactly 50 s after initial mixing. The percentage inhibition was calculated and reported to trolox activity.

Antioxidant Activity of the Extracts in a Cellular System. RAW 264.7 cells (1×10^6 cells per tube), a mouse macrophage-like cell line transformed with the Abelson leukemia virus, were incubated for 1 h in phosphate-buffered saline (PBS), pH 7.4, and 100 ng/mL PMA or 100 ng/mL bacterial LPS in the presence of different concentrations of the extracts, and the IC_{50} was calculated.

$O_2^{\bullet-}$ Generation. This was assayed by measuring the Cu−Zn SOD-inhibitable reduction of 0.15 mM cytochrome c (horse heart type VI) in the presence of 0.6 mM L-NIO (*19*).

NO Generation. Nitrites were measured as indicators of NO generation using Griess reagent (*19*), and assays were performed in the presence of 0.6 mM L-arginine and 60 U/mL Cu−Zn SOD. In addition, 0.6 mM L-NIO was used in some tubes to evaluate the degree of L-NIO inhibitable NO production.

Animals Studies with the Extracts. Swiss mice and Sprague−Dawley rats were kept at 22 ± 2 °C with 55 ± 10% relative humidity and controlled lighting (12 h light/dark cycle) throughout the study. Animals were fed with standard feedstuff. The diet and water were both consumed ad libitum. The procedures and animal care were performed in compliance with the appropriate laws and European Union guidelines. The Ethic Committee of the University of Barcelona approved the experiments.

Toxicity Studies. For the acute toxicity assay, we followed the up-and-down method according to OCDE 425 guidelines. The *F. vesiculosus* extracts were resuspended in 1% carboximethylcelulose and orally administered by intragastric canula or by an intraperitoneal route to rats and mice of both sexes. The intraperitoneal route was used to increase plasma and tissues levels of the compounds absorbed. An Irwin test (*20*) was performed at 1 and 5 h after administration of the extracts to detect motor, respiratory, temperature, circulatory, behavior, or other alterations. Animals were observed over a period of 7 days, and the approximate lethal dose 50 (LD_{50}, dose by which half of the animals died) was established.

A 4 week toxicity study was carried out in rats with an initial weight of 150 g. Five groups of seven males and seven females were daily administered either 1% carboximethylcelulose (control group) or extract 1 or 2 at two doses by an intragastric canula. Taking into account the approximate LD_{50} by oral route for rats, we chose 200 and 750 mg/kg as the low dose and the high dose, respectively, to be administered to the rats. This latter dose was used to observe the potential toxicity. Because of the characteristics of the extracts, it was not advisable to daily administer a higher dose. The food and water intake, as well as the body weight, were checked twice a week. At the end of the experimental period, rats were housed in individual metabolic cages

to collect urine and feces, fasted for 24 h, and anesthetized with chloral hydrate. The rats were then exsanguinated from the heart, and the most important organs were removed, weighed, and histologically examined. The hematological and clinical chemistry parameters evaluated were as follows:

In blood: WBC, RBC, platelet count, hemoglobin, hematocrit, MCH, MCHC, and MCV (counter Celltacα); prothrombine time, prothrombine activity, and cephaline time

In plasma: proteins, uric acid, urea, glucose and α-amylase (Biosystems); transaminases (Olympus); alkaline phosphatase, bilirubin, total cholesterol, and triglycerides (Randox); electrolytes such as Cl^-, Na^+, and K^+ (Easy Lyte); and osmolarity (MicroOsmometer model 3300)

In urine: diuresis; pH; excretion of Cl^-, Na^+, and K^+ (Easy Lyte); osmolarity (MicroOsmometer model 3300); and creatinine (Biosystems)

In feces: occult blood (*21*)

In tissues: Rats were perfused through the left ventricle with 4% formaldehyde in 0.1 M PBS. Organs were embedded in paraffin and cut. Obtained sections (7 μm thick) were stained with hematoxylin−eosin. Histopathological changes were evaluated, taking into account the cell types affected and the degree of alteration involved.

Phloroglucinol and Fucoxanthin Evaluation in Rat Plasma. Analyses were carried out using plasma from 24 h-fasted male rats daily treated with the two *F. vesiculosus* extracts (200 mg/kg body weight) for 4 weeks. Phloroglucinol was evaluated in plasma samples (100 μL) to which 100 μL of 1% HCOOH in methanol was added, and the resulting mixture was vortexed for 1 min. The mixture was centrifuged at 10000g for 1 min, and the upper layer was dried under N_2. The residue was dissolved in 50 μL of methanol:0.1% HCOOH in water (50:50, v/v), and 20 μL was injected into the HPLC-MS/MS system. The cone voltage was 30 V, the collision energy was 30 V, and argon was used at 2×10^{-3} mbar to improve fragmentation in the collision cell. The fragmentation transition for the multiple reaction monitoring experiment was $(m/z)^-$ 125→57, with a dwell time of 0.8 s. Phloroglucinol was used as the external standard, and its primary stock solution (1 mg/mL) was diluted to yield working solutions in methanol:0.1% HCOOH in water (50:50, v/v) in the range 5−50 ng/mL. The stock solution and the working solutions were stored at −80 and 4 °C, respectively.

Fucoxanthin was evaluated in plasma samples (100 μL) to which 200 μL of ethanol-containing echinenone as internal standard was added, and the resulting solution was extracted by 300 μL of hexane. The upper layer was dried under N_2, the residue was dissolved in 50 μL of methanol, and 20 μL was injected into the HPLC-MS/MS system. The cone voltage was 30 V, the collision energy was 35 V, and argon was used at 3.2×10^{-3} mbar to improve fragmentation in the collision cell. The fragmentation transitions for the multiple reaction monitoring experiment were as follows: $(m/z)^+$ 659→109, $(m/z)^+$ 641→109, and $(m/z)^+$ 581→109, with a dwell time of 0.2 s per transition. Fucoxanthin was used as an external standard, and its primary stock solution (1.13 μg/mL) was diluted to yield working solutions in methanol in the range 6.5−260 ng/mL. The stock solution and the working solutions were stored at −80 and 4 °C, respectively.

Nonenzymatic Protection in Rat Plasma. Tests were carried out using plasma from 24 h-fasted male rats daily treated by oral route with *F. vesiculosus* extracts (200 mg/kg body weight) at the end of the 4 week toxicity and also with plasma from male rats treated with 43 mg/kg body weight of phloroglucinol in similar conditions to *F. vesiculosus*-treated rats. The dose of phloroglucinol was chosen to administer a similar dose of polyphenols in the three groups of rats. These assays were performed only in males, as it was assumed that both sexes would respond similarly (*13*).

The reducing power was measured as previously described (*14*) although using 30% diluted plasma. The concentration of the total sulfydryl (−SH) groups was measured in 50 μL of plasma using glutathione as a positive control (*22*).

Oxidative stress induces lesions to cells and tissues and also to molecules and complex macromolecules such as low-density lipoproteins. Besides antioxidants, paraoxonase-1 (PON-1) present in high-density lipoproteins protects low-density lipoproteins against oxidation.

The PON-1 activity was determined using paraoxon as a substrate (*23*). Plasma was incubated at 25 °C in 100 mM Tris buffer, pH 8.0,

7776 *J. Agric. Food Chem.,* Vol. 56, No. 17, 2008

Zaragozá et al.

Table 1. Chemical Composition (g/100 g) of *F. vesiculosus* Extracts 1 and 2[a]

	extract 1	extract 2
water	5.3 ± 0.0	9.1 ± 0.0***
ashes	13.7 ± 0.1	13.0 ± 0.1**
proteins	3.6 ± 0.0	5.4 ± 0.0***
total lipids	1.2 ± 0.0	7.5 ± 0.6***
carbohydrates	17.8 ± 0.5	11.1 ± 0.4***
uronic acids	9.9 ± 0.4	10.1 ± 0.6
total fiber	20.1 ± 1.5	1.3 ± 0.1***
iodine (mg/kg)	113 ± 1	77 ± 2***

[a] Results are expressed as means ± SEM. Differences between extracts by the Student's *t* test are given by the following: **$p < 0.01$; ***$p < 0.001$; $n = 3$ replicates.

in the presence of 2 mM $CaCl_2$ and 2 mM paraoxon. Paraoxon degradation to *p*-nitrophenol was monitored by the absorbance increase at 412 nm (extinction coefficient 18290 L/mol/cm) for 5 min. One unit of PON-1 hydrolyzed 1 nmol of paraoxon/min.

To evaluate the $O_2^{\bullet-}$ scavenging activity, we used the same procedure (*15*) as described in the noncellular system, albeit with serial dilutions of plasma. An oxygen radical absorbance capacity (ORAC) assay was used to elucidate the antioxidant behavior of plasma against the peroxyl radicals with *R*-phycoerythrin serving as a redox-sensitive fluorescent indicator and ABAP as a chemical peroxyl radical generator (*24*). Trolox was used as a positive control. *R*-Phycoerythrin fluorescence decline was measured as the difference between 5 and 30 min at 37 °C (excitation wavelength, 530 nm; emission wavelength, 585 nm).

The thiobarbituric acid-reactive substances (TBARS) in plasma as indicative of malondialdehyde presence were determined by the method of Yagi (*25*) with one minor modification: Butylated hydroxytoluene and ethylenediaminetetraacetic acid were added to the reaction mixture as antioxidants at a final concentration of 0.01% and 1.3 μM, respectively.

The NO released in plasma was oxidized to nitrites. NO was measured in deproteinized samples (100 μL) by 20% trichloroacetic acid (1:1 v/v) at 4 °C and centrifuged at 18600*g* for 15 min. Supernatants (50 μL) were incubated in 5 mL of a solution (10 mM KI and 10 mM H_2SO_4) converting nitrites to NO, which was evaluated by a 2.0 mm Iso-NOP sensor. The maximal signal was recorded and compared to a $NaNO_2$ standard.

Enzymatic Protection in Rat Erythrocytes. Tests were carried out in erythrocytes from 24 h-fasted male rats daily treated by oral route with *F. vesiculosus* extracts (200 mg/kg body weight) at the end of the 4 week toxicity study and with phloroglucinol (43 mg/kg body weight) in the same conditions as *F. vesiculosus*-treated rats.

To evaluate the Cu−Zn SOD activity, 750 μL of hemolysed erythrocytes was used according to a technique based on the inhibition of pyrogallol auto-oxidation (*26*). The catalase activity was measured in 20 μL of hemolysed erythrocytes at room temperature, and the decomposition of H_2O_2 at 240 nm was evaluated (*27*). The rate constant ($k = 10^7$ L/mol s) for the first 30 s was calculated.

Statistical Analysis. The statistical analysis was conducted by using the SPSS version 12 statistical analysis packages. Results were expressed as the means ± standard errors of the mean (SEM). Data were evaluated using either the unpaired Student's *t* test or one-factor analysis of variance. The DMS test for multiple comparisons was used to detect differences among groups ($p < 0.05$).

RESULTS AND DISCUSSION

Characterization of the *F. vesiculosus* Extracts. Although extract 1 contained less proteins and lipids than extract 2, it was richer in carbohydrates and total fiber. The presence of total fiber in the extracts is inversely related to the percentage of ethanol used in the extraction procedure (**Table 1**). The iodine content (**Table 1**) was greater in extract 1 than in extract 2. The European Scientific Committee on Food suggests an upper intake of 600 μg of iodine/day whether the limit set by the Joint
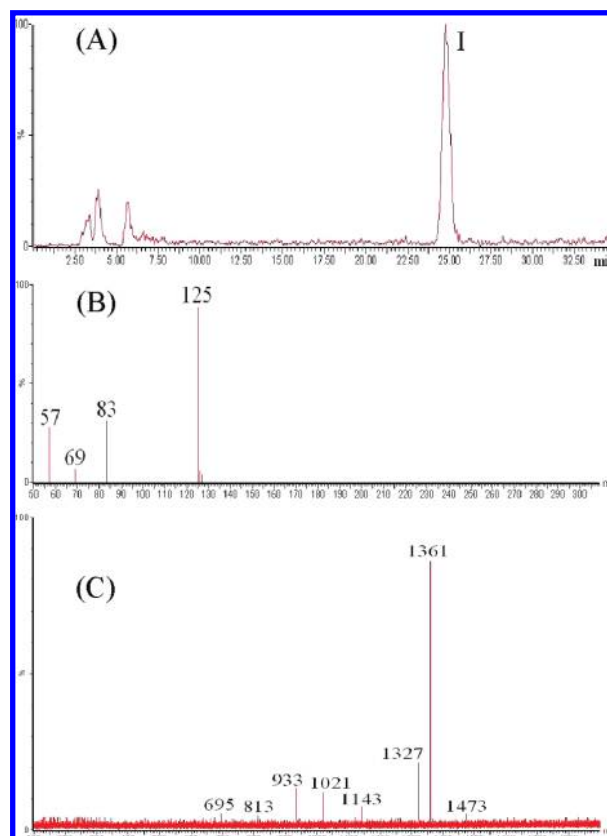

Figure 1. (**A**) LC-DAD-MS chromatogram of a *F. vesiculosus* extract. (**B**) Ions found under the peak I (RT = 26 min) using cone voltage values higher than 30 V. The ions with $(m/z)^-$ 125 correspond to phloroglucinol, while ions with $(m/z)^-$ 83, 69, and 57 were its main ions product. Phloroglucinol (RT = 5.6 min) was not found. (**C**) LC-MS/MS analysis in parent scan mode. The ions found correspond to the parents of ion products with $(m/z)^-$ 125.

Expert Committee on Food Additives is 1000 μg/day. A new European Union regulation (2006/352) sets to 150 μg/day the maximum iodine allowed in food supplements. Thus, the iodine content in the extracts should be taken into account in order not to exceed the limits allowed when administered to humans.

Total polyphenols (g/100 g extract) expressed as gallic acid and phloroglucinol equivalents were higher in extract 1 (28.8 ± 1.7 and 27.7 ± 1.5, respectively) than in extract 2 (18.0 ± 1.6 and 16.3 ± 0.3, respectively) (**Table 2**). The molecular masses of the polyphenolic fractions of the two extracts demonstrate that they were similar with about 40% > 300 kDa, 43% between 50 and 300 kDa, 7% between 30 and 50 kDa, 10% between 10 and 30 kDa, and traces with molecular masses <10 and <3 kDa. The LC-DAD-MS(MS) chromatograms of the *F. vesiculosus* extract 2 are reported in **Figure 1**. The UV spectrum of peak I (**Figure 1A**) suggests a structure of a phloroglucinol derivative, while the mass spectrum at high cone voltage values yields ions with $(m/z)^-$ 125, 83, 69, and 57 (**Figure 1B**). The ions with $(m/z)^-$ 83, 69, and 57 represent ions product of phloroglucinol, suggesting that peak I may contain a phloroglucinol moieties. **Figure 1C** reports the LC-MS/MS chromatogram obtained when acquiring the parents of ions product with $(m/z)^-$ 125. It should be emphasized that peak I was not present in the fraction lower than 10 kDa and that phloroglucinol was not found in the *F. vesiculosus* extracts. Thus, the results seem to indicate that the polyphenolic fractions are high molecular weight forms of polymeric phloroglucinol. Although there were differences in the condensed forms,

Toxicity and Antioxidant Activity of *Fucus vesiculosus*

J. Agric. Food Chem., Vol. 56, No. 17, 2008 **7777**

Table 2. Polyphenols and Fucoxanthin in *F. vesiculosus* Extracts 1 and 2[a]

		extract 1	extract 2
total polyphenols	g gallic ac. equiv/100 g	28.8 ± 1.7	18.0 ± 1.6**
	g phloroglucinol equiv/100 g	27.7 ± 1.5	16.3 ± 0.3**
fucoxanthin	mg/100 g	0.26 ± 0.02	1.24 ± 0.06***

[a] Results are expressed as means ± SEM. Differences between extracts by the Student's *t* test are given by the following: **$p < 0.01$; ***$p < 0.001$; $n = 3$ replicates.

structures, and molecular weights, the chemical properties were similar. Thus, it was difficult, and also not particularly useful, to evaluate single phlorotannins in the two extracts. Moreover, monomeric forms of polyphenols were not present.

Although fucoxanthin levels were low in both extracts, these levels were higher in extract 2 (1.24 ± 0.06 mg/100 g) (**Table 2**). The extracts also contained astaxanthin and violaxanthin in low concentrations (<0.05 mg/100 g).

Antioxidant Activity of the Extracts in Noncellular Systems. The two extracts from *F. vesiculosus* showed a good activity in a broad spectrum of the antioxidant chemical tests. Both extracts had important reducing power (**Table 3**); thus, they are likely to contain molecules that act as electron donors, converting them into more stable products, thereby terminating the radical chain reaction. Although this approach is being used in the evaluation of antioxidant activity of dietary polyphenols, caution is necessary to extrapolate the results to the physiological environment, as this assay is performed in acidic pH value.

Extract 1 proved to be a more potent antioxidant in terms of a variety of radical-generating systems such as $O_2^{\bullet-}$ (381%), DPPH radical (218%), peroxyl radicals (71%), and also in the ABTS radical cation (80%) than did extract 2 (**Table 3**). The $O_2^{\bullet-}$ scavenging activity is relevant because although this radical does not directly initiate lipid oxidation, but it does generate hydroxyl radicals by the Fenton reaction. No assays on the antioxidant activity of *F. vesiculosus* have been performed to date. However, assays using other brown seaweeds rich in phloroglucinol or in phloroglucinol derivatives showed great reducing power and DPPH radical scavenging activity (*7*) and $O_2^{\bullet-}$ (*8, 9*) and DPPH radical scavenging activities (*8*), respectively. Azo compounds such as ABAP generate peroxyl radicals at a reproducible and constant rate. Phenols are the most common antioxidant compounds to readily scavenge peroxyl radicals by donating hydrogen atoms as well as by stabilizing the resulting antioxidant radical by electron delocalization and/ or intramolecular hydrogen bonding or by further oxidation (*28*). The higher activity of extract 1 is likely to be related to the greater content of total polyphenols. In addition, polyphenols and lipophilic antioxidants such as carotenoids can be measured with the ABTS radical cation test. Fucoxanthin has also been described to be a scavenger of DPPH (*10, 11*) and ABTS radicals (*11*). The higher levels of fucoxanthin present in extract 2 should be partly responsible for the antioxidant activity (*10*).

The capacity of the two extracts from *F. vesiculosus* to scavenge $O_2^{\bullet-}$, peroxyl radicals, or artificial radicals provides support for antioxidant efficacy in vitro. However, the cell-based in vitro results, as well as those obtained ex vivo after administering the extracts, are considered more predictive than the present results based on chemically generated oxidants.

Antioxidant Activity of the Extracts in a Cellular System. Activated inflammatory cells, such as macrophages, contain NAD(P)H-oxidase. The NAD(P)H-oxidase and the inducible NO synthase are strongly induced upon exposure to bacterial endotoxin and inflammatory cytokines. These enzymes mediate the massive production of $O_2^{\bullet-}$ and NO, respectively, which

serve as ingredients for the formation of highly pro-oxidant radical species such as hydroxyl radical and peroxynitrite (a potent oxidant generated by the mol:mol reaction of $O_2^{\bullet-}$ and NO) (*29*) that lead to severe damage to host cells and tissues. The extracts had a similar effect on reducing $O_2^{\bullet-}$ production when RAW 264.7 macrophages were stimulated with PMA (**Table 4**). Indeed, extract 1 proved to be more effective than extract 2 when LPS was used (**Table 4**). With regard to decreasing NO production, extract 1 was more effective than extract 2 (**Table 4**). A reduction of NO production by RAW 264.7 macrophages by phloroglucinol derivatives was also observed by Ishii et al. (*30*). Furthermore, our group has previously demonstrated that NO inhibits the $O_2^{\bullet-}$ production by inflammatory exudate-derived polymorphonuclear leukocytes (*31*) by inactivating the NAD(P)H oxidase. Thus, it is likely that the final $O_2^{\bullet-}$ measurement may be the result of NAD(P)H inactivation by NO and the $O_2^{\bullet-}$ scavenging activity, which is supported by the reduction of IC_{50} in the X/XO assay. Reduced $O_2^{\bullet-}$ and NO production can indicate that the generation of either hydroxyl radicals or peroxynitrite will be reduced. The capacity of the two extracts to protect against oxidative damage caused by free radicals may be of great interest in the prevention of the acute and chronic inflammatory processes that occur in many diseases and are concomitant with the activation of polymorphonuclear leukocytes and macrophages. However, these cells also have an antimicrobial function because of their phagocytic nature and capacity to release reactive species into the phagosome. The measurement of $O_2^{\bullet-}$ and NO production by RAW 264.7 macrophages indicated that extract 1 exhibited higher antioxidant activity than extract 2, which may be related to the 60% higher polyphenols or polyphenols with higher antioxidant activity.

As observed with extracts from seaweed species other than *F. vesiculosus* obtained by 50% ethanol, the greater total polyphenol content correlates with greater $O_2^{\bullet-}$ scavenging activity in vitro (*9*). However, evidence of antioxidant activity in vitro cannot be easily extrapolated into an in vivo setting due to the fact that once ingested polyphenols and carotenoids undergo extensive modification during first-pass metabolism and also because of the concentrations tested (*32*).

Toxicity Studies. The LD_{50} by the oral route in rats for extracts 1 and 2 (**Table 5**) ranged between 1000 and 2000 and >2000 mg/kg, respectively, while with female mice, they ranged between 1000 and 2000 and >750 mg/kg, respectively. As expected, administration of the two extracts by an intraperitoneal route resulted in higher toxicity than by the oral route. Moreover, the extracts were more toxic in mice than in rats (**Table 5**). The Irwin test did not show important alterations even by the intraperitoneal route.

Treated female rats ingested approximately less food (13% reduction along the 4 week toxicity study) than their respective control; thus, their weight gain was also reduced. The hematological values did not reveal any pathological modification and were found to range within the normal limits for rats. We only detected a decrease in the white cell count in those groups treated with extract 2 (about 50%) and in the group treated with the high dose of extract 1 (about 30%). However, the differential white cell percentage was maintained. No differences were detected in any of the coagulation parameters assayed.

We have also observed an increase of α-amylase in rats treated either with the high dose of extract 1 (44%) or with both doses of extract 2 (about 36%), although these values were within the normal limits for rats. In addition, we observed an increase in Na^+ urine excretion when extracts 1 and 2 (68 and

Table 3. Reducing Power and Antioxidant Activities of *F. vesiculosus* Extracts 1 and 2 by Noncellular Systems[a]

	extract 1	extract 2	phloroglucinol
reducing power (mmols quercetin equiv/g extract)	1.5 ± 0.1 b	1.0 ± 0.0 a	8.5 ± 0.1 c
$O_2^{\bullet-}$ scavenging activity (IC$_{50}$, μg/mL)	182 ± 7 b	694 ± 35 c	86 ± 4 a
DPPH (IC$_{50}$, μg/mL)	11.9 ± 0.1 a	26 ± 1 c	18.0 ± 0.9 b
TRAP (mmols trolox equiv/g extract)	2.4 ± 0.1 a	1.4 ± 0.1 a	28.7 ± 1.8 b
ABTS scavenging activity (mmols trolox equiv/g extract)	1.8 ± 0.1 b	1.0 ± 0.1 a	17.0 ± 0.2 c

[a] Significant differences were evaluated by the DMS test. Mean values within a row with unlike superscript letters were significantly different, $p < 0.05$. Results are expressed as means ± SEM; $n = 3$ replicates.

Table 4. $O_2^{\bullet-}$ and NO Production by *F. vesiculosus* Extracts 1 and 2 in RAW 264.7 Macrophages[a]

	inducible agent	extract 1	extract 2
$O_2^{\bullet-}$ (IC$_{50}$, μg/mL)	PMA	38 ± 4	31 ± 4
	LPS	39 ± 5	68 ± 6**
NO (IC$_{50}$, μg/mL)	PMA	37 ± 5	not inhibition
	LPS	95 ± 10	>100

[a] Results are expressed as means ± SEM. Differences between extracts by Student's *t* test are given by the following: **$p < 0.01$; $n = 4$ replicates.

Table 5. Approximate LD$_{50}$ Values of *F. vesiculosus* Extracts 1 and 2

			LD$_{50}$ (mg/kg body weight)	
animal	route	sex	extract 1	extract 2
rat	oral	female	1000−2000	>2000
		male	1000−2000	>2000
	i.p.	female	250	>500
		male	1000−2000	500
mice	oral	female	1000−2000	<750
		male	1000	500
	i.p.	female	150−200	250−500
		male	150−200	250−500

90%, respectively) were administered at the highest dose. However, we have not observed a decrease in plasma Na$^+$ concentration. This may be explained by an effect of extracts on Na$^+$ release from the metabolism of organic molecules such as uronic acids, as NaCl was not present in the extracts. Fecal blood yielded negative results throughout the 4 week of daily oral doses of *F. vesiculosus* extracts, indicating an absence of lesions in the gastrointestinal tract.

In terms of organ weight (**Table 6**), we found an increase in the relative liver weight in male rats treated with the high dose of extract 2 (25%) and in the kidneys of male rats treated with the high dose of extract 1 (21%). Increased liver weight stemmed not from fat accumulation, since this was not observed in the anatomopathological study, nor was it due to alcohol, as the latter was absent from extracts. Moreover, the absence of any changes in liver and kidney functional parameters (transaminases, alkaline phosphatase, and bilirubin and urea and creatinine, respectively) supports a lack of toxicity in these organs. In general, the anatomopathological changes observed were slight or moderate and neither matched the severity of acute alterations nor were dose-dependent, and they were slightly greater in rats treated with extract 2. Small alterations in both extract treated rats groups were observed in lungs that may be related to a hypersensitivity reaction stemming from factors other than extract administration. The overall results obtained from the 4 week toxicity study indicate that even at the daily dose of 750 mg/kg body weight for 4 weeks, no relevant signs of toxicity occurred with the two *F. vesiculosus* extracts studied.

Phloroglucinol and Fucoxanthin Evaluation in Rat Plasma. Phloroglucinol and fucoxanthin studied by HPLC-MS/MS, in their either parent or metabolized forms, were not found in rat plasma following the daily 4 week treatment at 200 mg/kg, probably due to their low systemic uptake (*32*), and, thus, small reached concentrations.

Nonenzymatic Antioxidant Protection in Rat Plasma. Knowledge-based antioxidant efficacy must drive from in vivo/ex vivo data, and its activity can be through a direct scavenging of free radicals. The increased reducing power (29%), PON-1 activity (33%), and $O_2^{\bullet-}$ scavenging activity (25%) in the plasma of rats treated with the extract 2 (**Table 7**) indicate that some compounds were absorbed and their presence in the parent or metabolized forms will exhibit antioxidant activity in the organism although the −SH groups were maintained at levels similar to those of the control. An increase in the reducing power has also been observed in rats a short time after the administration of a very high single dose (2000 mg/kg containing 600 mg/kg polyphenols) of brown seaweeds extract (VNP) (*7*). The fact that plasma nitrite concentration remained constant while plasma exhibited a higher $O_2^{\bullet-}$ scavenging activity may indicate that less peroxynitrite is being produced. This observation is corroborated by the significantly reduced levels of TBARS (17%) (**Table 7**), which most likely reflects not only plasma's increased ability to scavenge free radicals but also PON-1's greater hydrolytic activity. PON-1 protects low-density lipoproteins from oxidative modification by reactive oxygen species and thus significantly contributes to the atheroprotective effect of high-density lipoproteins. It hydrolyzes phospholipid hydroperoxides and cholesterol ester hydroperoxides (esterase activity) and reduces lipid hydroperoxides to the respective hydroperoxides as well as degrades hydrogen peroxide (peroxidase activity) (*23*). Thus, antioxidants in extract 2 maintain ex vivo their reducing capacity and $O_2^{\bullet-}$ scavenging activity, in addition to other activities. It is likely that the increased activity of extract 2 ex vivo is caused by a distinct phlorotannin content and to the presence of fucoxanthin, as carotenoids react with peroxyl radicals by forming an adduct.

Enzymatic Antioxidant Protection in Rat Erythrocytes. The Cu−Zn SOD activity was increased (about 32%) in rats treated with the extract 2 and phloroglucinol (**Figure 2A**). The catalase activity (**Figure 2B**), however, remained in both groups of extract-treated rats at levels similar to those of the control group. An increased Cu−Zn SOD activity can be seen as an additional protective mechanism against oxidative stress if catalase activity is sufficiently high enough to counteract the H_2O_2 generated by the SOD. Depending upon the phenolic compound involved (its structure, specificity to the tissue, and the degree of systemic bioavailability), its effect on rat antioxidant enzymes is likely to be different (*33*).

Our results indicate that the oxidative stress observed in plasma and erythrocytes is reduced when rats are treated with the extract 2 for a period of 4 weeks, although extract 1 contained 60% more total polyphenols than extract 2, which is probably a reflection of absorption and the rate of metabolism and also of the levels of fucoxanthin, although compounds were not detected. One possibility is that the antioxidant effects ex

Table 6. Body and Relative Organ Weights of Female Rats after a Daily Dose of *F. vesiculosus* Extracts 1 and 2 during a 4 Week Period[a]

	body weight	heart	stomach	liver	spleen	lungs	kidneys	ovaries/testes
females								
control	211 ± 6 a	0.44 ± 0.02 a	1.07 ± 0.08 a	3.41 ± 0.25 a	0.30 ± 0.01 a	0.68 ± 0.06 a	0.54 ± 0.03 a	0.15 ± 0.03 a
extract 1, 200 mg/kg	201 ± 9 a	0.43 ± 0.03 a	0.94 ± 0.07 a	3.79 ± 0.07 a	0.34 ± 0.02 a	0.88 ± 0.23 a	0.51 ± 0.02 a	0.13 ± 0.01 a
extract 1, 750 mg/kg	190 ± 5 a	0.44 ± 0.03 a	1.01 ± 0.03 a	3.79 ± 0.38 a	0.34 ± 0.02 a	0.70 ± 0.10 a	0.49 ± 0.03 a	0.13 ± 0.01 a
extract 2, 200 mg/kg	209 ± 4 a	0.41 ± 0.01 a	1.03 ± 0.05 a	3.41 ± 0.05 a	0.29 ± 0.01 a	1.06 ± 0.21 a	0.51 ± 0.02 a	0.15 ± 0.02 a
extract 2, 750 mg/kg	196 ± 8 a	0.43 ± 0.02 a	1.05 ± 0.05 a	3.53 ± 0.22 a	0.34 ± 0.02 a	0.67 ± 0.13 a	0.55 ± 0.02 a	0.17 ± 0.04 a
males								
control	302 ± 10 a	0.40 ± 0.01 a	0.84 ± 0.04 a	3.16 ± 0.13 a	0.24 ± 0.01 a	0.63 ± 0.14 a	0.48 ± 0.02 a	0.73 ± 0.08 a
extract 1, 200 mg/kg	299 ± 8 a	0.42 ± 0.01 a	0.91 ± 0.07 a	3.63 ± 0.20 ab	0.31 ± 0.05 a	0.59 ± 0.02 a	0.52 ± 0.02 ab	0.75 ± 0.05 a
extract 1, 750 mg/kg	285 ± 6 a	0.41 ± 0.00 a	0.93 ± 0.05 a	3,28 ± 0.19 a	0.28 ± 0.01 a	0.64 ± 0.03 a	0.59 ± 0.02 b	0.85 ± 0.05 a
extract 2, 200 mg/kg	295 ± 6 a	0.43 ± 0.02 a	0.98 ± 0.04 a	3.42 ± 0.10 a	0.26 ± 0.01 a	0.60 ± 0.03 a	0.55 ± 0.02 ab	0.76 ± 0.02 a
extract 2, 750 mg/kg	270 ± 5 a	0.44 ± 0.02 a	0.97 ± 0.03 a	3.97 ± 0.19 b	0.31 ± 0.02 a	0.82 ± 0.11 a	0.57 ± 0.04 ab	0.90 ± 0.07 a

[a] Significant differences were evaluated by DMS test. Mean values within a column with unlike superscript letters were significantly different, $p < 0.05$. Results are expressed as means ± SEM; $n = 7$ rats.

Table 7. Oxidative Stress Parameters in Plasma of Male Rats after a Daily Dose (200 mg/kg) of *F. vesiculosus* Extracts 1 and 2 during a 4 Week Period[a]

	control	extract 1	extract 2	phloroglucinol
reducing power (mM quercetin equiv)	139 ± 12 a	148 ± 12 a	180 ± 13 b	182 ± 13 b
−SH groups (mM glutathione equiv)	0.23 ± 0.02 a	0.26 ± 0.02 a	0.26 ± 0.02 a	0.24 ± 0.02 a
PON-1 activity (U/mL)	179 ± 7 a	214 ± 12 bc	238 ± 17 c	200 ± 11 ab
$O_2^{\bullet-}$ scavenging activity (IC$_{50}$, μg/mL)	45 ± 4 b	39 ± 4 ab	34 ± 3 a	42 ± 4 ab
ORAC (mM trolox equiv)	2.2 ± 0.1 a	2.7 ± 0.2 a	2.7 ± 0.2 a	2.8 ± 0.2 a
NO concentration (μM)	2.9 ± 0.2 a	3.0 ± 0.2 a	2.9 ± 0.2 a	3.1 ± 0.2 a
TBARS (μM)	8.2 ± 0.5 b	7.9 ± 0.4 ab	6.8 ± 0.5 a	7.2 ± 0.6 ab

[a] Significant differences were evaluated by DMS test. Mean values within a row with unlike superscript letters were significantly different, $p < 0.05$. Results are expressed as means ± SEM; $n = 7$ rats.

vivo are mediated by metabolites with also biological activity. It is also likely that a synergic effect among polyphenols (*34*) or carotenoids (*35*), in their either parent or metabolized forms, takes place.
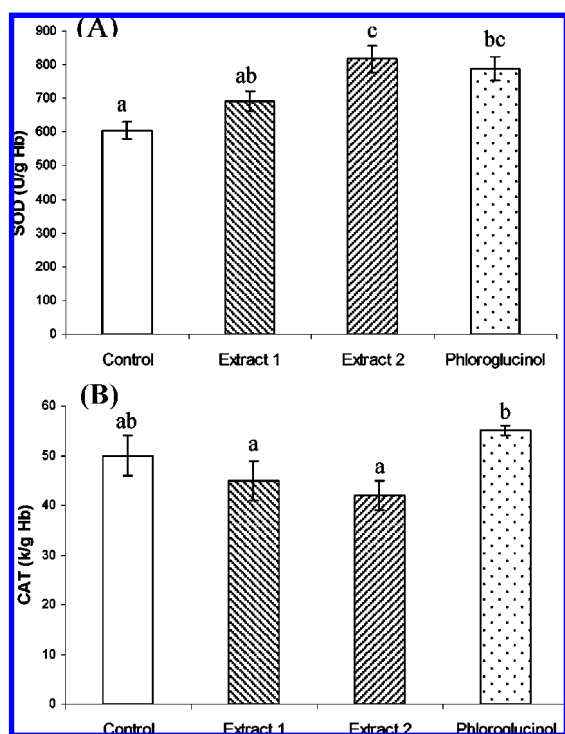

Figure 2. Enzymatic antioxidant activities in erythrocytes from male rats after a daily dose (200 mg/kg) of *F. vesiculosus* extracts 1 and extract 2 during a 4 week period. (**A**) Cu−Zn SOD. (**B**) Catalase. Data are expressed as means ± SEM. Significant differences were evaluated by DMS test, $p < 0.05$. Data with different letters are significantly different; $n = 7$ rats.

This paper confirms that antioxidant efficacy can be demonstrated in one system but fails to protect in others. These observations reflect the distinct mechanism of action between the two seaweed extracts. In conclusion, here, we provide evidence that the two extracts obtained from *F. vesiculosus* lack toxicity and exhibit important antioxidant activity against chemically generated oxidants and in vitro. However, only extract 2 shows an antioxidant activity ex vivo through preventing oxidant formation, scavenging $O_2^{\bullet-}$ and reducing active intermediates. Our findings are relevant, as they elucidate the potential future application of the *F. vesiculosus* extract 2 (Healsea) both as functional foods and as food supplements. Although extrapolation of findings from animal experiments to humans is difficult, it is conceivable that *F. vesiculosus* extracts will maintain the antioxidant activity in humans.

ABBREVIATIONS USED

$O_2^{\bullet-}$, superoxide anion; NO, nitric oxide; DAD, diode array detection; X/XO, xanthine/xanthine oxidase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABAP, 2,2′-azo-bis-2-amidinopropane; ABTS, 2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); SOD, superoxide dismutase; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; L-NIO, N5-(1-iminoethyl)-L-ornithine dihydrochloride; IC$_{50}$, inhibitory concentration 50; TRAP, total radical antioxidant parameter; LD$_{50}$, lethal dose 50; PON, paraoxonase; ORAC, oxygen radical absorbance capacity; TBARS, thiobarbituric acid-reactive substances.

LITERATURE CITED

(1) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933–956.

(2) Krinsky, N. I.; Kyung-Jin, Y. Carotenoid-radical interactions. *Biochem. Biophys. Res. Commun.* **2003**, *305*, 754–760.

(3) Kuo, S. M.; Leavitt, P. S.; Lin, C. P. Dietary flavonoids interact with trace metals and affect metallothionein level in human intestinal cells. *Biol. Trace Elem. Res.* **1998**, *62*, 135–153.

(4) Robak, J.; Gryglewski, R. J. Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol.* **1998**, *37*, 837–841.

(5) Cook, N. C.; Samman, S. Flavonoids. Chemistry, metabolism, cardioprotective effects, and dietary source. *J. Nutr. Biochem.* **1996**, *7*, 66–76.

(6) Ragan, M. A.; Glombitza, K. W. Phlorotannins, brown algal polyphenols. *Progr. Phycol. Res.* **1986**, *4*, 129–241.

(7) Kang, K.; Park, Y.; Hawng, H. J.; Kim, S. H.; Lee, J. G.; Shin, H. C. Antioxidative properties of brown algae polyphenolics and their perspectives as chemopreventive agents against vascular risk factors. *Arch. Pharm. Res.* **2003**, *26*, 286–293.

(8) Lee, S. M.; Na, M. K.; An, R. B.; Min, B. S.; Lee, H. K. Antioxidant activity of two phloroglucinol derivatives from *Dryopteris crassirhizoma. Biol. Pharm. Bull.* **2003**, *26*, 1354–1356.

(9) Nakai, M.; Kageyama, N.; Nakahara, K.; Miki, W. 2006. Phlorotannins as radical scavengers from the extract of *Sargassum ringgoldianum. Marine Biotechnol.* **2006**, *8*, 409–414.

(10) Yan, X.; Chuda, Y.; Suzuki, M.; Nagata, T. Fucoxanthin as the major antioxidant in *Hijikia fusiformis*, a common edible seaweed. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 605–607.

(11) Sachindra, N. M.; Sato, E.; Maeda, H.; Hosokawa, M.; Niwano, Y.; Kohno, M.; Miyashita, K. Radical scavenging and singlet oxygen quenching activity of marine carotenoid fucoxanthin and its metabolites. *J. Agric. Food Chem.* **2007**, *55*, 8516–8522.

(12) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

(13) Verhagen, H.; Auroma, O. I.; VanDelft, J. H. M.; Dragsted, L. O.; Ferguson, L. R.; Knasmüller, S.; Pool-Zobel, B. L.; Poulsen, H. E.; Williamson, G.; Yannai, S. The 10 basic requeriments for a scientific paper reporting antioxidant, antimutagenic or anticarcinogenic potential of test substances in in vitro experiments and in animal studies in vivo. *Food Chem. Toxicol.* **2003**, *41*, 603–610.

(14) Oyaizu, M. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. *Jpn. J. Nutr.* **1986**, *44*, 307–315.

(15) Ukeda, H.; Maeda, S.; Ishii, T.; Sawamura, M. Spectrophotometric assay for superoxide dismutase based on tetrazolium salt 3′-{1-[(phenylamino)-carbonyl]-3,4-tetrazolium}-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate reduction by xanthine-xanthine oxidase. *Anal. Biochem.* **1997**, *251*, 206–209.

(16) Gerhäuser, C. Mechanism-based in vitro screening of potential cancer chemopreventive agents. *Mutat. Res.* **2003**, *523−524*, 163–172.

(17) Visioli, F.; Galli, C. Evaluation of antioxidant capacity by chemiluminescence. *Anal. Biochem.* **1997**, *249*, 244–246.

(18) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. A. Antioxidant activity applying an improved ABTS radical cation decolourization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231–1237.

(19) Ródenas, J.; Mitjavila, M. T.; Carbonell, T. Simultaneous generation of nitric oxide and superoxide by inflammatory cells in rats. *Free Radical Biol. Med.* **1995**, *18*, 869–875.

(20) Irwin, S. Comprehensive observational assessment: Ia. A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. *Psychopharmacology (Berlin)* **1968**, *13*, 222–257.

(21) Needhan, C. D.; Simpson, R. G. The benzidine test for occult blood in faces. *Q. J. Med.* **1952**, *21*, 123–133.

(22) Ellman, G.; Lysko, H. A precise method for the determination of whole blood and plasma sulfhydryl groups. *Anal. Biochem.* **1979**, *93*, 98–102.

(23) Aviram, M.; Hardak, E.; Vaya, J.; Mahmood, S.; Milo, S.; Hoffman, A.; Billicke, S.; Draganov, D.; Rosenblat, M. Human serum paraoxonases (PON1) Q and R selectively decrease lipid peroxides in human coronary and carotid atherosclerotic lesions: PON1 esterase and peroxidase-like activities. *Circulation* **2000**, *101*, 2510–2517.

(24) Glazer, A. N. Phycoerythrin fluorescence-based assay for reactive oxygen species. *Methods Enzymol.* **1990**, *186*, 161–168.

(25) Yagi, K. Assay for blood plasma or serum. *Methods Enzymol.* **1984**, *105*, 328–331.

(26) Marklund, S. L. Pyrogallol autooxidation. In *Handbook of Methods for Oxygen Radical Research*; Greenwald, R. A., Ed.; CRC Press: Boca Ratón, FL, 1985; Vol. 24, pp 3−247.

(27) Aebi, H. E. Catalase in vitro. *Methods Enzymol.* **1984**, *105*, 121–126.

(28) Frankel, E. N.; Meyer, A. S. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *8*, 1925–1941.

(29) Liochev, S. I.; Fridovich, I. Superoxide and nitric oxide: consequences of varying rates of production and consumption: A theoretical treatment. *Free Radical Biol. Med.* **2002**, *33*, 137–141.

(30) Ishii, R.; Masakazu, H.; Koichi, S.; Munehisa, A.; Susumu, K. Inhibitory effects of phloroglucinol derivates from *Mallotus japonicus* on nitric oxide production by a murine macrophage-like cell line, RAW 264.7, activated by lipopolysaccharide and interferon-γ. *Biochim. Biophys. Acta* **2001**, *1568*, 74–82.

(31) Ródenas, J.; Mitjavila, M. T.; Carbonell, T. Nitric oxide inhibits superoxide production by inflammatory polymorphonuclear leukocytes. *Am. J. Physiol.* **1998**, *274*, C827–C830.

(32) Kroon, P. A.; Clifford, M. N.; Crozier, A.; Day, A. J.; Donovan, J. L.; Manach, C.; Williamson, G. How should we assess the effects of exposure to dietary polyphenols in vitro. *Am. J. Clin. Nutr.* **2004**, *80*, 15–21.

(33) Breinholt, V.; Lauridsen, S. T.; Dragsted, L. O. Differential effects of dietary flavonoids on drug metabolism and antioxidant enzymes in female rats. *Xenobiotica* **1999**, *29*, 1227–1240.

(34) Pignatelli, P.; Ghiselli, A.; Buchetti, B.; Carnevale, R.; Natella, F.; Germanó, G.; Fimognari, F.; Di Santo, S.; Lenti, L.; Violi, F. Polyphenols synergistically inhibit oxidative stress in subjects given red and white wine. *Atherosclerosis* **2006**, *188*, 77–83.

(35) Stahl, W.; Junghans, A.; de Boer, B.; Driomina, E. S.; Briviba, K.; Sies, H. Carotenoid mixtures protect multilamellar liposomes against oxidative damage: synergistic effects of lycopene and lutein. *FEBS Lett.* **1998**, *427*, 305–308.