Journal of Functional Foods 60 (2019) 103399

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Two compounds of the Colombian algae *Hypnea musciformis* prevent oxidative damage in human low density lipoproteins LDLs

Gladys Rozo[a,*], Claudia Rozo[a], Mónica Puyana[a], Freddy A. Ramos[b], Carmen Almonacid[c], Henry Castro[d]

[a] Facultad de Ciencias Naturales e Ingeniería, Universidad Jorge Tadeo Lozano, Carrera 4 No. 22-61, Bogotá, Colombia
[b] Departamento de Química, Facultad de Ciencias, Universidad Nacional de Colombia-Sede Bogotá, Carrera 30 No.45-03, Bogotá, Colombia
[c] Facultad Ciencias de la Salud, Universidad Colegio Mayor de Cundinamarca, Calle 28 No 5B-02, Colombia
[d] Research Group on Food Chemistry, Departamento de Química, Universidad Nacional de Colombia, Carrera 45 No. 26-85, Bogotá, Colombia

ARTICLE INFO

Keywords:
Algae
Lipidic peroxidation
Epicatechin
Phloretin
Supercritical fluid extraction

ABSTRACT

People of the Caribbean believe that red algae have properties that are beneficial to human health. We aimed to test the beneficial properties of *Hypnea musciformis* by measuring its ability to prevent oxidation in human LDLs. We obtained algal extracts via supercritical fluid extraction using 15 different combinations of pressure, temperature and solvent polarity. We quantified phenol contents and measured antioxidant activity through ABTS and TBARS assays. The ability of each extracted compound to protect LDLs against oxidative damage was compared with that of natural vitamin E. The extracts obtained under low pressure (10–20 MPa) with 8% ethanol provided 42% extra protection, while those obtained under high pressure (30 MPa) with 8% ethanol provided 62.5% extra protection. The compounds responsible for this activity were purified through chromatography and identified by ^{1}HNMR spectroscopy as (−)-epicatechin and phloretin. Our results emphasize the high potential of extracts of *H. musciformis* as functional foods.

1. Introduction

Algae or seaweed are considered “healthy foods” due to their low content of lipids and high concentrations of polysaccharides, unsaturated fatty acids, vitamins A, B1, B12, C, D and E, riboflavin, niacin, pantothenic and folic acid and minerals such as calcium, phosphorus, sodium, iodine, potassium and trace elements (Cardozo et al., 2007; Lordan, Ross, & Stanton, 2011). Algae are also important sources of compounds with a wide spectrum of biological activities, including antioxidant, antimicrobial, antitumor and antiviral properties (de Almeida et al., 2011; Wijesekara, Pangestuti, & Kim, 2011). There are at least 400 patents based on algae or their derivatives with applications in cosmetics, medicine and as chemotherapeutic agents, among others (Cardozo et al., 2007; D'Orazio et al., 2012; Lee et al., 2013). In the Caribbean region, several species of red algae are used to prepare energizing beverages, which are also believed to present aphrodisiac properties (Gordon, 2001; Radulovich, Umanzor, & Cabrera, 2013) These beverages derive from ancient cultural traditions from the Caribbean peoples (Radulovich et al., 2015)

The base ingredients in the preparation of these drinks are agar extracted from *Hydropuntia cornea*, *H. crassisima*, *Gracilaria domingensis* and *Gelidium serrulatum* and carrageenan from *Eucheuma isiforme* (Murcia & Alanis, 2003).

Despite the use of several red algae in the Caribbean region, the nutraceutical and phytopharmaceutical properties of these species have not been determined. Therefore, to propose any of these algae as a functional food, the effect of food components on target functions relevant to human health and well-being must be determined (Andrade et al., 2013; Cardozo et al., 2007; Darias-Jerez, 2016; Nagai & Yukimoto, 2003). Significant antioxidant activity is attributed to algae, with consequent prevention of cell damage, aging and cardiovascular diseases by inhibiting oxidative reactions that cause oxidative stress and tissue damage (Batista, Charles, Mancini-filho, & Vidal, 2009; Blunt et al., 2009; Domínguez, 2013).

One of the cardiovascular diseases that may be prevented by consuming a diet rich in antioxidants is atherosclerosis (Gutiérrez-

Abbreviations: FDA, food and drug administration; LDLs, low-density lipoproteins; SFE, supercritical fluid extraction; ABTS, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; TPC, total phenol content; TBARS, thiobarbituric acid-reactive substances assay; CER, constant extraction rate; FER, falling extraction rate period; DCR, diffusion controlled rate

* Corresponding author.
E-mail address: gladys.rozo@utadeo.edu.co (G. Rozo).

https://doi.org/10.1016/j.jff.2019.06.001
Received 28 January 2019; Received in revised form 31 May 2019; Accepted 1 June 2019

Rodríguez, Juárez-Portilla, Olivares-Bañuelos, & Zepeda, 2018; Li et al., 2014). Atherosclerosis is a disease characterized by the progressive enlargement of the arterial walls due to the accumulation of intracellular lipids. This process leads to the formation of plaques limiting blood flow, which can in turn cause tissue damage and myocardial infarction (Bayod, Villarroel, Peréz Lorenz, & Puzo, 2013). Atherosclerosis is the most frequent cause of death in western countries (Fishbein & Fishbein, 2015; Hernández-Leiva, 2011; Mendivil, Sierra, & Pérez, 2004). Although many factors can increase the risk of developing this disease, a positive correlation between the oxidation of low-density lipoproteins (LDLs) and the development of atherosclerosis has been established (Hulthe & Fagerberg, 2002; Siti, Kamisah, & Kamsiah, 2015). The consumption of low levels of antioxidants may cause LDL oxidation and degradation (Combs, 2008; Rice-Evans, Miller, & Paganga, 1997; Witztum & Hörkkö, 1997), hence, the cholesterol esters that are usually transported by LDL accumulate as nonsoluble residues, leading to the obstruction of arterial walls (Salvayre, Negre-Salvayre, & Camaré, 2016). Low levels of antioxidants in blood are also correlated with obesity, reduced immune function and even mental health complications (Laguerre, Lecomte, & Villeneuve, 2007; Sánchez-Muniz, Bocanegra de Juana, Bastida, & Benedí, 2013). Antioxidant activity has been reported in brown algae (Chakraborty, Praveen et al., 2013; Nohynek, Antignac, Re, & Toutain, 2010; Thomas & Kim, 2011), green algae (Echavarría, Franco, & Martínez, 2009) and red algae (Agatonovic-Kustrin, Kustrin, Angove, & Morton, 2018)

According to Food and Drug Administration (FDA) guidelines, to propose a food for the potential reduction of suffering from a disease, it is mandatory to describe the components and substances present in that food that are responsible for that particular desired effect (Winklhofer-Roob, Faustmann, & Roob, 2017). In the case of antioxidants from algae, it is necessary to provide scientific evidence that some of the compounds in the alga indeed play an antioxidant role and are effective in preventing the oxidation of LDL in human blood.

H. musciformis is abundant at various shallow locations in the Colombian Caribbean, and some of our previous work showed that its extracts present antioxidant activity, as determined by the DPPH method (unpublished data). Therefore, in this work, we wanted to determine which compounds are responsible for the antioxidant activity of *H. musciformis* extracts and evaluate whether those compounds can prevent the oxidation of human LDL.

2. Materials and methods

2.1. Algae collection

Samples of *Hypnea musciformis* were collected along the rocky shoreline of Santa Marta Bay, Colombian Caribbean (11°7′33.94″N, 74°13′58.50″W) in November 2015. The material was cleaned of epiphytes and rinsed with distilled water. We kept some samples in a 5% v/v solution of formaline in sea water to be used for species identification under the criteria reported by Littler, Littler, Bucher, and Norris (1989) and Dawes and Mathieson (2008)

Once in the laboratory, samples were dried in a recirculating oven until constant weight (less than 0.1% of water in the sample). After milling and sieving, we obtained particles ranging between 0.3 and 0.5 mm in diameter. This size is within the range required for extraction procedures (Asep et al., 2008; Pereira & Meireles, 2010). Ground algal samples were stored in darkness at room temperature (15 °C).

2.2. Extraction of bioactive components

Powdered algal samples of 25.00 ± 0.06 g each were used for extraction with supercritical fluid (SFE) in dynamic mode, with carbon dioxide and ethanol as the cosolvents (EtOH/CO_2). To identify the optimal set of parameters, we conducted the extractions under three different conditions regarding the temperature (40°, 50° and 60 °C), pressure (10, 20 and 30 MPa) and solvent (mixture of EtOH with CO_2 at 2%, 5% and 8%) and all the corresponding combinations. Details of the extraction parameters for each sample are shown in Table 1.

Table 1
Supercritical fluid extraction conditions for powdered samples of the red alga *Hypnea musciformis*.

Extract*	Conditions		
	Pressure (MPa)	Temperature (°C)	EtOH %
A	10	40	2
B	20	50	2
C	10	60	2
D	30	40	2
E	30	60	2
F	10	50	5
G	20	40	5
H	20	60	5
I	30	50	5
J	10	40	8
K	10	60	8
L	20	50	5
M	20	50	8
N	30	40	8
O	30	60	8

* 15 different extracts were obtained and labeled with letters reflecting the various extraction conditions.

We defined a preliminary set of parameters to optimize the time of extraction. We conducted the extraction procedure at 20 MPa with a flow of 0.4 kg/h to identify the minimal time required to obtain enough extract to be used in the following procedures. We used the same extraction time for the three different pressures.

Extracts were collected at the following established intervals: every 5 min for 30 min, every 10 min for 60 min, every 30 min for 150 min and every 60 min for 450 min (Benelli, Riehl, Smânia, Smânia, & Ferreira, 2010). The extracts were then concentrated under rotary evaporation and stored in the freezer under total darkness.

2.3. Screening of antioxidant activity in vitro

The extracts were evaluated to identify and select those with the greatest antioxidant activity to be further tested *in vitro*. The following assays were performed: analysis of total phenol content (TPC), decolorization of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and the TBARS assay, which measures substances formed as byproducts of lipid peroxidation that are reactive to thiobarbituric acid. All spectrophotometric readings were carried out in a Thermo Scientific Evolution 300 spectrophotometer. Spectrophotometric curves were analyzed with VISION PRO software. Each assay was performed with five replicates. Once the most promising extracts were detected, they were run in an *in vitro* assay to assess their role in preventing the oxidation of human LDLs. The compounds responsible for LDL protection were separated using standard chromatographic techniques and identified through the analysis of ^{1}H nuclear magnetic resonance (H ^{1}NMR) spectra.

2.3.1. Determination of the total phenol content (TPC)

The TPC of each extract was determined following Kumar, Tayade, Chaurasia, Sunil, and Singh (2010). This assay determines the total reducing capacity of a sample based on modification of the Folin-Ciocalteu method of phenol oxidation. TPC was expressed as equivalents of gallic acid (GAE) mg/g per sample.

2.3.2. Determination of concentrations in the thiobarbituric acid-reactive substance assay (TBARS assay)

This assay measures the potential protective effect of the tested

substances against lipid oxidation. In this case, fragments of lyophilized mussels (*Perna viridis*) were used as a lipid source. The assay was carried out using two different methodologies. The first method was performed following Chakraborty, Joseph et al. (2013), with some modifications. The algal extracts were resuspended in 1 mL of ethanol (to a final concentration of the extract between 0.1 and 0.6 mg/mL) and incubated with 10 mg of lyophilized mussel, 2 mL of acetic acid (density 1.03 g/mL) and 2 mL of an aqueous solution of thiobarbituric acid (TBA, 0.78 g/100 mL) at 95 °C for 45 min. This mixture was cooled at room temperature and centrifuged (8000 r/min for 10 min) to obtain the supernatant. The absorbance of the supernatant was registered at 532 nm in a spectrophotometer. The antioxidant capacity was expressed as mmol/L of malonaldehyde/kg of sample. The effective antioxidant concentration was calculated with an external standard curve based on malonaldehyde (MDA).

The second method consisted of determining the concentration of MDA, a product formed by the breakdown of polyunsaturated fatty acids, which react with TBARS to form a fatty acid complex that can be measured spectrophotometrically at 535 nm (Tsikas, 2017). Degradation products were quantified using a standard curve based on tetraethoxypropane (dimethylacetal-TMP or diethylacetal-TEP) as a source of MDA (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002).

2.3.3. Quantification of antioxidant activity via the ABTS assay

This assay measures the ability of antioxidants present in the extracts to reduce the cationic radical $ABTS^{*}$, formed by the oxidation of ABTS with potassium persulphate. This assay was performed following Re et al. (1999) with minor modifications. We reacted 7 mM ABTS with 2.45 mM potassium persulphate over a 16-hour period to produce the $ABTS^{*}$ radical. Subsequently, 3 mL of this radical was added to the reaction cell, and 10 µL of the sample was added to determine the amount of antioxidants. The test temperature was maintained at 30 °C. Absorbance readings were taken every 20 s for seven minutes at 730 nm.

2.4. Oxidation of human low density lipoproteins (LDLs)

LDL was obtained following Esterbauer and Striegl (1989) with minor modifications. A blood sample was obtained from a young, healthy, nonsmoking, male donor who had not consumed any antioxidants. We used a commercially available SER-PAK kit to measure the baseline levels of serum cholesterol, HDL, LDL-cholesterol and triglycerides from the LDL donor, and we confirmed that these parameters were consistent with the clinical report of a healthy male between 18 and 25 years of age. LDL samples from the donor's blood were obtained via precipitation with heparin at a 1:1 ratio (blood-heparin) using Ecoline reagent at 100,000 U/L. The sample was then centrifuged at 26.000 rpm for 30 min at room temperature. The resulting precipitate consisted of pure LDL that was reconstituted in phosphate buffer at a pH of 7.4. Samples were stored at −70 °C prior to performing the oxidation experiments.

LDL samples were activated for oxidation by adding a 20 µM/L solution of Cu^{+2} in the presence of the antioxidant extract. The fatty acids present in the LDL sample were continuously measured by spectrophotometric absorption at 234 nm at a constant temperature of 37 °C. Prior to the assay, the algal extracts were resuspended in dimethyl sulfoxide (DMSO) at a concentration equivalent to 40 µM/L of α-tocopherol. α-tocopherol acetate (Vitamin E) was used as an antioxidant standard at a concentration of 40 µM/L (Maiorino, Zamburlini, Roveri, & Ursini, 1995).

2.5. Isolation and structural elucidation of compounds **1** and **2**

2.5.1. Isolation and purification

The extracts that exhibited the greatest antioxidant activity and best responses regarding the protection of LDL were purified via flash column chromatography using 60 Merck (230–400 Mesh) silica gel as the stationary phase. We conducted our assays using two different mixtures as eluents: ethanol-ethyl acetate, with increasing polarity from 80:20 to 30:70, followed by chloroform-methanol, with polarity from 100:0 to 70:30.

Purity was monitored by thin-layer chromatography (TLC) using Merck silica gel TLC-plates 60 HF_{254}. The results were revealed with iodine vapors, UV light (254 and 365 nm) and αα diphenyl-ß-picrylhydrazyl (DPPH) (Hostettmann, Gupta, Marston, & Ferreira Quiróz, 2008; Sakamoto, Hatakeyama, Ito, & Handa, 2012). The solvents employed for chromatographic separations were of analytical grade. High-performance liquid chromatography (HPLC) was employed for purity monitoring with an ultrafast liquid chromatograph Shimadzu Prominence (Maryland, CA, EUA) coupled to an LCMS-2020 selective mass detector.

Monitoring of the chromatographic system and data acquisition and processing were all performed using Lab Solutions software version 3.5.

The chromatographic conditions for these analyses were set with a Shim Pack column (6 cm × 2 mm diameter, particle size 2.1 µm and stationary phase C18). Several gradient elution systems were developed using LiChrosolv (Merck) solvents for the reversed-phase system. The injection volume was 55 µL; the column temperature was 40 °C; and the flow rate for the mobile phase was 0.6 mL/min. In addition, the following interface and spectrometer conditions were applied: ESI (electrospray ionization) mode with a flow rate of the drying gas of 15 L/min and a nebulizer gas rate of 1.5 L/min. The temperatures for the heating block and the solvent elimination line were 300 °C and 350 °C, respectively. Initially, the analyses were conducted simultaneously in SCAN mode (+and -) with the purpose of ion detection. Subsequently, the analyses were performed in positive and negative SIM (selective ion monitoring) mode. The voltage applied to the capillary varied from 1 to 4.5 kV and −1 to −4.5 kV.

2.5.2. Structural elucidation of compounds responsible for protection against LDL oxidation

Following the separation and purification of the compounds responsible for protection against LDL oxidation, structural elucidation was performed using Nuclear Magnetic Resonance H^1NMR. The NMR spectra were captured in a Bruker Advance 400 (400.13 MHz) spectrometer using methanol-d_4 (Merck, Darmstadt, Germany) as the solvent, with a deuteration grade of 99.8%. The solvent residual signals were used as an internal standard (δ_H 7.26, δ_C 77.0 for chloroform; δ_H 3.31 and 4.87, δ_C 49.1 for methanol).

To differentiate the isomers present in the samples that were previously separated, purified and identified, optical rotations were measured in a Polartronic E, Schmidt + Haensch polarimeter using a cell of 1 mL × 5 cm length at 20 °C. All samples subjected to optical rotations were resuspended in a 1% acetone water mix in a 1:1 proportion (50% v/v).

2.6. Statistical analysis of data

All statistical analyses were carried out with the software R 3.0.3 (R Core Team, 2016). All assays were performed with five repetitions. The data obtained were expressed as the mean ± standard deviation. One-way analyses of variance (ANOVA) and post hoc Tukey tests were used to compare means. The significance level was set at a p-value ≤ 0.05.

3. Results and discussion

3.1. Extraction of bioactive components via the SFE method

The extraction curve for the supercritical fluid extraction of a dried, ground sample of *H. musciformis* is presented in Fig. 1.

The constant extraction rate period (CER) was identified between 0 and 80 min. During this period, 70% (104 mg) of the extract was obtained using EtOH with CO_2 at 5% p/p. During this first step, the extract

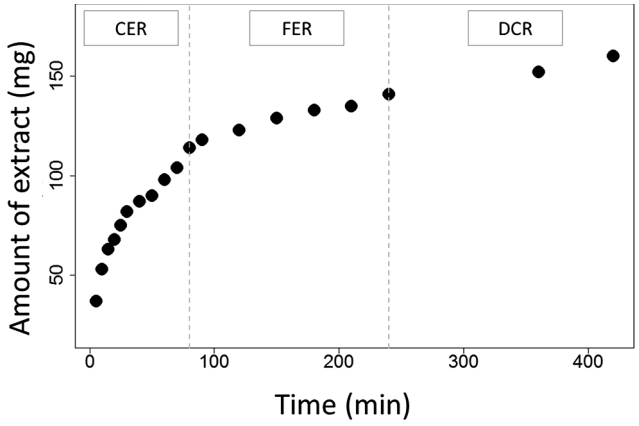

Fig. 1. Extraction curve for the supercritical fluid extraction of a dry and ground sample of the alga *Hypnea musciformis* using 5% ethanol in CO_2. CER: Constant Extraction Rate, between 0 and 80 min correspondent to the period of easy migration from the extract to the solvent. FER: Falling Extraction Rate period; between 80 and 210 min, when the speed of extraction is driven by diffusion processes DCR: Diffusion Controlled Rate, between 240 and 420 min, when the diffusion and extraction reach the equilibrium.

can be obtained easily because the CO_2 and ethanol dissolve the nonpolar molecules located on the surface of each algal particle as a result of convective processes. The falling extraction rate period (FER) occurred between 80 and 210 min. During this period, the extraction rate fell to 21% (31 mg) of the total algal dry weight. This decline occurred because diffusion processes control mass transfer, meaning that the solvent is in excess outside the particles and scarce inside. Therefore, it can penetrate the algal particles via diffusion and dissolve the nonpolar molecules inside. Then, the resultant solution must migrate to the exterior of the particles (Kumar et al., 2010; Mendiola, Herrero, Castro, & Ibáñez, 2013). This is the reason that the extraction becomes very slow. Finally, the diffusion-controlled rate period (DCR) occurred between 240 and 420 min, with a final yield of 9% (13 mg) of the extract. The asymptote in the extraction curve (DCR) is a result of the solvent and product concentrations being homogeneous inside and outside the particles (Kumar et al., 2010; Mendiola et al., 2013). After 240 min of extraction, 93.92% of the extract was obtained considering all extraction phases, the CER, the FER and a portion of the DCR. Extraction times beyond 240 min were not considered, since at 420 min, the extraction rate was very low (0.70%, 148 mg).

Once the time conditions were established, supercritical fluid extractions (SFEs) were performed. All extractions were carried out in a period of 240 min. The varying conditions were pressure, temperature and the percentage of ethanol (2–8%) used for extraction (Table 2). Each extraction was performed in triplicate.

The results show that variations in pressure and temperature allowed the solvent to behave similarly to a fluid of greater polarity, increasing the extraction yield. Hence, compounds with medium and high polarity should be expected in extracts J, K, M, N and O. We observed that the greatest extraction yield was obtained for extract N (30 MPa, 40 °C, 8% EtOH), followed by extract O (30 MPa, 60 °C, 8% EtOH) and extract K (10 MPa, 60 °C, 8% EtOH), for which the highest concentration of ethanol as a cosolvent was used. For *H. musciformis,* an extraction yield between 2.6 and 4.8% was obtained by maceration with dichloromethane or methanol, respectively (Chakraborty, Joseph et al., 2013; Guedes et al., 2013). Extraction yields also depend on solvent polarity and chemical differences related to collection areas (Chakraborty, Praveen et al., 2013; Guedes et al., 2013). In our case, the addition of ethanol improved solubility in supercritical CO_2, increasing the extraction efficiency. Ethanol interacts with the matrix and substances to be extracted in a way that encourages rapid desorption into the supercritical fluid, achieving a more efficient extraction (Asep, Jinap, Jahurul, Zaidul, & Singh, 2013).

Table 2
Extraction performance in the experimental supercritical fluid extraction (SFE) of dried samples of the alga *Hypnea musciformis*.

Extract*	Experimental Conditions			Extract Yield
	Pressure	Temperature	EtOH	%
	(MPa)	(°C)	%	
A	10	40	2	0.22 ± 0.01
B	20	50	2	0.74 ± 0.01
C	10	60	2	0.23 ± 0.01
D	30	40	2	0.35 ± 0.01
E	30	60	2	0.6 ± 0.01
F	10	50	5	0.11 ± 0.01
G	20	40	5	0.63 ± 0.01
H	20	60	5	0.68 ± 0.01
I	30	50	5	0.93 ± 0.01
J	10	40	8	0.98 ± 0.01
K	10	60	8	1.06 ± 0.01
L	20	50	5	0.68 ± 0.01
M	20	50	8	0.82 ± 0.01
N	30	40	8	1.24 ± 0.01
O	30	60	8	1.13 ± 0.01

All extractions were carried out in a period of 240 min. The varying conditions were pressure, temperature and the percentage of ethanol (2–8%) used for extraction. Each extraction was performed in triplicate. Yield values are expressed as the average ± standard deviation (sd).

3.2. Antioxidant potential of SFE extracts of H. musciformis

The total polyphenol content and the antioxidant activity of all SFE extracts of *H. musciformis* were assessed. Antioxidant activity was determined through the ABTS and TBARS assays (Table 3).

An increase in the proportion of ethanol during extraction resulted in an increase in the antioxidant activity of the extract. Extracts labeled with the letters M, N and O showed the greatest antioxidant activity as determined in the ABTS inhibition assay (Fig. 2). The combination of the extraction parameters can modify not only the amount of extract obtained from the sample but also the properties of the extract because

Table 3
Determination of total polyphenol content and evaluation of antioxidant activity in SFE extracts of *Hypnea musciformis.*

Extracts	TPC mg GAE/g alga	ABTS % RSA	TBARS *"Perna viridis"* (MDAEC/kg)	TBARS micromol MDA/kg
A	0.897 ± 0.005	1.47 ± 0.820	6.44 ± 0.726	2.64 ± 0.562
B	2.695 ± 0.420	1.74 ± 0.848	6.19 ± 0.150	2.56 ± 0.691
C	0.861 ± 0.083	2.87 ± 3.599	7.15 ± 0.943	3.41 ± 0.201
D	2.012 ± 0.014	9.32 ± 0.143	4.36 ± 0.828	5.16 ± 0.326
E	2.146 ± 0.131	3.29 ± 0.101	4.03 ± 0.657	5.40 ± 0.144
F	1.961 ± 0.065	4.94 ± 1.659	8.64 ± 0.616	4.53 ± 0.021
G	2.108 ± 0.158	6.30 ± 0.591	8.06 ± 0.952	4.56 ± 0.037
H	1.051 ± 0.057	6.41 ± 0.156	6.18 ± 0.169	3.33 ± 0.661
I	1.986 ± 0.070	8.04 ± 0.729	6.58 ± 0.420	5.46 ± 0.338
J	0.999 ± 0.014	10.52 ± 0.460	11.27 ± 0.776	9.68 ± 0.591
K	2.949 ± 0.048	12.86 ± 1.268	14.24 ± 1.110	9.05 ± 0.015
L	2.956 ± 0.061	15.37 ± 1.741	5.84 ± 0.527	2.18 ± 0.007
M	3.095 ± 0.019	16.10 ± 1.342	12.08 ± 0.437	12.56 ± 1.829
N	3.425 ± 0.046	17.43 ± 1.350	18.23 ± 0.859	20.23 ± 0.815
O	3.786 ± 0.116	16.22 ± 0.832	19.54 ± 0.498	17.67 ± 0.732

Antioxidant activity was assessed through the ABTS and TBARS assays. Data are expressed as the means ± sd. TBARS *Perna viridis:* TBARS assay using *Perna viridis* mussels as a lipid source. MDAEC/kg: Antioxidant activity expressed as equivalent mM of malondialdehyde per kilogram of sample. RSA: Radical scavenging ability. MDA: malonaldehyde. All assays were performed in quintuplicate. Data are expressed as the means ± standard deviation (sd).

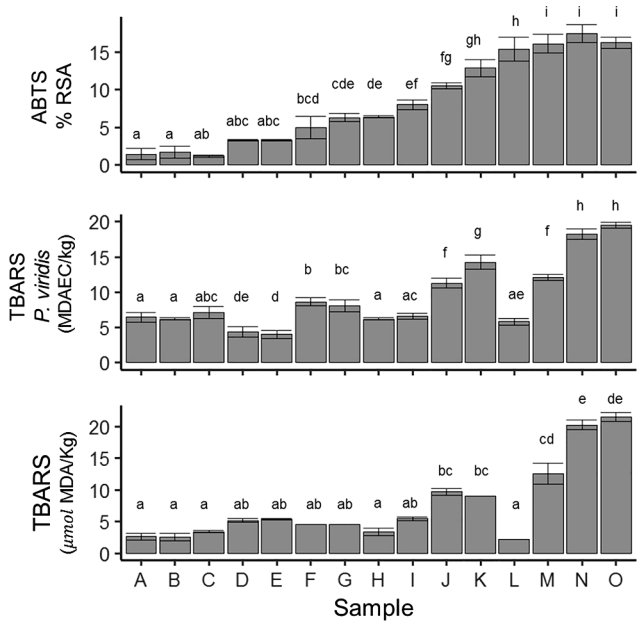

Fig. 2. Antioxidant activity of extracts of *Hypnea musciformis* as determined by TBARS by using *Perna viridis* mussel as lipid sources. TBARS assay and ABTS assay. The groups with different letters are different according to a Tukey HSD test. The X axis is the same for the three graphs in this figure.

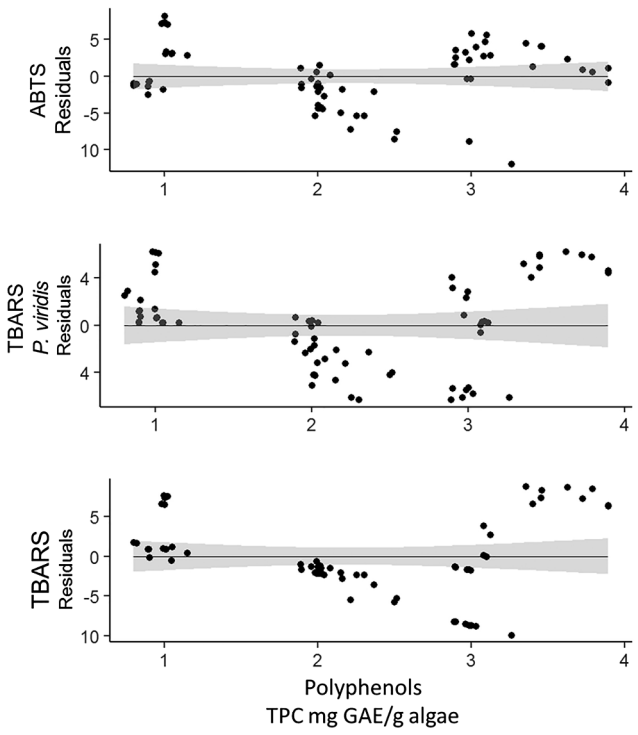

Fig. 3. Residuals of the correlations between antioxidant activity and total amount of polyphenols in extract of *H. musciformis*. For the three methods used, the correlation between the antioxidant activity and polyphenols was significant. For ABTS (top) $Y_{ABTS} = 4.42x - 1.3$, $p\ value = 1.69 \times 10^{-12}$, $R^2 = 0.49$. For TBARS *Perna viridis*, $Y_{TBARSP.viridis} = 3.04x + 2.57$, $p\ value = 1.52 \times 10^{-8}$, $R^2 = 0.35$. For TBARS $Y_{TBARS} = 4.12x - 1.57$, $p\ value = 8.27 \times 10^{-10}$, $R^2 = 0.40$. The presence of values outside of the confidence interval around the regression (gray) indicates other antioxidant compounds or synergic interactions. The X axis is the same for the three graphs in this figure.

varying the extraction parameters will mean that the compounds present in the extracts slightly different. These differences are sufficient to modify the antioxidant activity of the extract. Thus, we correlated the bioactivity of the extracts with the extraction parameters through multivariate ANOVA. We repeated this analysis for each of the methods used to measure bioactivity.

For the ABTS assay, the ANOVA test was significant, with an R^2 of 0.95 and a p-value $< 2.2 \times 10^{-16}$ All the variables (pressure, temperature and EtOH concentration) and their interactions were significant. These results indicate that there is an optimal combination between the parameters for obtaining the extracts with the highest level of bioactivity, which in general involved a temperature of 50 °C and medium to high pressure. However, the effect of EtOH was stronger than those of the other variables, considering that increasing the concentration of ethanol from medium (5%) to high (8%) roughly doubled the antioxidant activity in all cases (data not shown).

For the TBARS with *P. viridis* assay, the ANOVA result was significant, with an R^2 of 0.98 and a p-value $< 2.2 \times 10^{-16}$. In this case, the effect of each of the three variables was important, as were those of their interactions. The most important factor for optimizing antioxidant activity was again the amount of ethanol. Increasing the ethanol concentration in the solvent from medium (5%) to high (8%) increased the antioxidant activity of the obtained extracts from 50 to approximately 300% depending on the combination of the other two variables (temperature and pressure) (data not shown).

For TBARS, the ANOVA result was significant, with R^2 of 0.84 and p-value $< 2.2 \times 10^{-16}$. In this case, the effect of each of the three variables was important, as were those of their interactions. The best combination for obtaining an extract with high antioxidant activity was high pressure (30 MPa) with either low (40 °C) or high (60 °C) temperature (but not medium temperature, 50 °C). Again, increasing the ethanol concentration in the solvent from medium (5%) to high (8%) increased antioxidant activity by approximately 300% (data not shown)

Extracts J, K, M, N and O exhibited the greatest potential antioxidant activity in the three assays (Fig. 2). The best extracts in terms of their biological activity were obtained with methods involving a higher proportion of ethanol as a cosolvent.

In this study, two different TBARS assays were performed because TBARS may react with alkenes, certain proteins and degradation products (Gordon, 2001; Shahidi & Zhong, 2005)

Extracts M, N, and O were the most active in inhibiting thiobarbituric acid as a way to evaluate antioxidant activity.

There was a correlation between the antioxidant activity and the amount of polyphenols in the samples between the three methods evaluated (residuals and confidence intervals are shown in Fig. 3).

For ABTS, $Y_{ABTS} = 4.42x - 1.3$, $p\ value = 1.69 \times 10^{-12}$, $R^2 = 0.49$. For TBARS with *Perna viridis*, $Y_{TBARSP.viridis} = 3.04x + 2.57$, $p\ value = 1.52 \times 10^{-8}$, $R^2 = 0.35$. For TBARS, $Y_{TBARS} = 4.12x - 1.57$, $p\ value = 8.27 \times 10^{-10}$, $R^2 = 0.40$. The observed correlation, however, explained only approximately 30% of the sample variability, as shown in the residuals of our linear regressions in Fig. 3. Although it is possible that a greater concentration of polyphenols in the sample increases the antioxidant potential of the extracts, there might be other factors involved in this variability, such as the presence of carotenoids and other pigments produced by red algae (Pangestuti & Kim, 2011).

Phenol concentrations in *H. musciformis* are highly variable and are highly dependent on the extraction method and the inherent biological and ecological parameters of the algae. The total phenol concentration in *H. musciformis* from the SE coast of Tamil Nadu, India, was found to be 6.91 mg EAG/g when extracted in methanol (Balamurugan & Selvam, 2013). The efficiency of phenol extractions is directly related to temperature increases (Chakraborty, Joseph et al., 2013). For the same algal species extracted with ethanol, phenol concentrations of 0.21 ± 0.04 mg GAE/g have been reported (Sarojini, Sujatha, & Santha Rao, 2016). Solvent mixtures of lower polarity, such as chloroform methanol at 2:1, have also been used, yielding low

concentrations of total polyphenols (0.58 ± 0.03 mg GAE/g) in *Gracilaria* sp. from the Marmara coast in Turkey (Yildiz, Vatan, Çelikler, & Dere, 2011). Although the concentration and structure of individual phenolic compounds is highly dependent on the geographical origin of the sample and the applied extraction procedures, we obtained a total polyphenol content (with a maximum of 3.786 ± 0.116 mg GAE/g for extract O) that was far greater than that reported from other samples of *H. musciformis* (Balamurugan & Selvam, 2013; Stengel, Connan, & Popper, 2011). This result may be attributed to other nonphenolic compounds producing positive readings with the Folin-Ciocalteu reagent given that, in addition to phenolic compounds, this technique can detect oxidized compounds that do not have phenolic groups in their structure (Stengel et al., 2011).

In summary, in SCF extraction, parameters such as pressure, temperature and the proportion of cosolvent (ethanol) are important for obtaining extracts with high antioxidant activity. However, our results indicate that the best yields and greater antioxidant activity were detected in those extracts obtained using 8% ethanol as a cosolvent.

Extracts M, N and O showed the greatest potential to inhibit the ABTS radical and the thiobarbituric acid reaction (Fig. 2). On the other hand, in the TBARS trial with *Perna viridis*, the most active extracts were J, K, M, N and O (Fig. 2).

Extracts J, K, M, N and O presented the greatest yields and promising antioxidant activity. Therefore, they were selected to be assayed as inhibitors of LDL oxidation. Since neither extracts J and M nor extracts N and O showed any significant differences in their antioxidant activity (Fig. 2), they were pooled to be tested *in vitro*, where they were referred to as extracts MJ, NO and K.

3.3. Protection of LDLs against oxidative damage

Prior to this assay, blood chemistry analysis of the blood donor showed an adequate level of LDLs for performing the assay (Table 4). The total cholesterol level was adjusted to 0.20 mg cholesterol/ml in pH 7.4 phosphate buffer.

Table 5 shows the results of the inhibitory potential of extracts of *H. musciformis* against human LDL oxidation.

Multiple comparative analyses showed significant differences in the inhibition of LDL oxidation by the different extracts of *H. musciformis*. LDLs treated with extract NO were 62.5% more effectively protected from oxidation than LDLs treated with vitamin E (p-value < 0.005). It takes just six molecules of alpha-tocopherol (vitamin E) to protect the nearly 5000 lipid molecules of a single particle of LDL against oxidation (Perez, 2007).

Hence, a comparison between treated and untreated LDLs showed that extract K and vitamin E were very mild protectors against LDL oxidation (23% and 33%, respectively) compared to extracts MJ (89.3%) and NO (116.7%).

Previous studies have correlated antioxidant activity in *H. musciformis* from South Korea to the total phenol content of this alga (Siriwardhana et al., 2008). Other studies have shown that algal consumption is related to the changes in consumers lipid profiles (Sánchez-Muniz et al., 2013). Our study allowed us to determine that extracts from *H. musciformis* may protect LDLs from oxidation between 40 and 60% more effectively than vitamin E.

In the kinetic curves of lipid oxidation, three characteristic phases are observed (Shahidi & Zhong, 2015; Winklhofer-Roob et al., 2017). Fig. 4 shows the kinetic curves of lipid oxidation for untreated LDLs and those treated with extracts MJ and NO or with vitamin E. Phase 1, referred to as the lag or latency phase, corresponds to the protection period during which LDLs are protected by antioxidants. In this phase, there is only a slight increase in absorbance due to the formation of a conjugate detected at 234 nm. Phase 2 corresponds to the propagation phase, during which large quantities of unsaturated fatty acids are formed. Phase 3 corresponds to the decay of all antioxidants present in the LDLs. An increase in the lag or latency phase is associated with an increased LDL protection capacity of the antioxidant (Shahidi & Zhong, 2015).

Table 6 includes the latency phase duration for each algal extract that was protective against LDL oxidation. Latency period times were determined from the slope intercept in the lag phase and that of the propagation phase curve (n = 5).

Latency periods correspond to the lag phase (phase 1) in the kinetic curve of lipid peroxidation. In this phase, LDLs are protected from oxidation. Extract concentrations were equivalent to 40 μM/L of alpha tocopherol (vitamin E).

The lag phase for untreated LDLs may be associated with natural endogenous or enzymatic antioxidants or with those present in the diet (Lönn, Dennis, & Stocker, 2012; Mirończuk-Chodakowska, Witkowska, & Zujko, 2018; Yoshida & Kisugi, 2010). In this case, LDLs were protected for a time frame of 72 ± 6 min, after which rapid oxidation began. This time frame was almost doubled by the addition of extract MJ or NO.

An enhanced protective effect of extracts from *H. musciformis* compared to the protective action of vitamin E was also detected. Extract NO increased the latency period by 76%, whereas extract MJ increased the latency period by 53%. The total polyphenol content of extracts M, J, N and O was high (Table 3). Therefore, the high antioxidant activity of the algal extracts may be partly due to the presence of substances with hydroxyl groups that display antioxidant action by neutralizing free radicals upon donating a hydrogen atom or an electron (Chakraborty, Joseph et al., 2013; Ibrahim et al., 2017; Maqsood, Benjakul, & Shahidi, 2013; Sonani, Rastogi, & Madamwar, 2017).

3.4. Structural elucidation of compounds **1** and **2**

Extracts MJ and NO were suspended in ethanol. Bioguided fractionation of extract MJ yielded compound **1** in the $CHCl_3$:MeOH at 80:20 fraction. This compound was of a deep reddish-brown color. Bioguided fractionation of extract NO yielded white compound **2** from the fraction that was soluble in ethanol:ethyl acetate at 30:70. Both compounds were tested for purity using RP-HPLC with acetonitrile:water (1:5 v/v). Compound **1** showed a signal at a retention time of 15.87 min with some minor signals. Compound **2** showed a signal at a retention time of 21.06 min with some minor signals.

Compounds were identified on the basis of their H^1NMR data compared with those recorded in the literature (Ananingsih, Sharma, & Zhou, 2013; Richard, Temsamani, Cantos-Villar, & Monti, 2013). The structure corresponding to compound **1** is presented in Fig. 5 (dataset Rozo et al., 2019) and agrees with the chemical structure of a catechin.

Table 4
Donor blood chemistry analysis prior to evaluation of the inhibitory potential of extracts of *Hypnea musciformis* against human LDL oxidation.

	Cholesterol mg/dL *mean* ± *sd*	Triacyl glycerides mg/dL *mean* ± *sd*	HDL mg/dL *mean* ± *sd*	LDL mg/dL *mean* ± *sd*	Protein g/L *mean* ± *sd*
Donor	150 ± 21	125 ± 28	51 ± 9	80 ± 22	54 ± 14
Reference* values for healthy males	150–220	112	> 45	< 110	60–80

HDL: High-density lipoproteins. LDL: Low-density lipoproteins. Data are expressed as the means ± standard deviations (sd).
* Values recovered from (Villalpando, Alvarez, & Gómez, 2017).

Table 5
Lipid peroxidation rate (minutes) of human LDL oxidation after incubation with extracts of *Hypnea musciformis* to assess their potential as oxidation inhibitors.

LDL trials	Untreated	Enriched with vitamin E	Incubation with *H. musciformis* extracts		
			MJ	NO	K
Lag phase (min)	72 ± 4.2[a]	96 ± 7.1[b]	136.3 ± 21.7[c]	156.0 ± 16.7[d]	86 ± 10.9[ab]
Increase in oxidation inhibition percentage		33.3	89.3	116.7	23.1
Increase in oxidation inhibition percentage compared to vitamin E			42.0	62.5	−7.7

All values are expressed as the mean ± standard deviation (SD), (n = 5); p-value < 0.005. Superscripted letters indicate statistically significant differences between treatments.
There were no significant differences in the protective potential of extract K compared to untreated LDLs (p-value < 0.005).

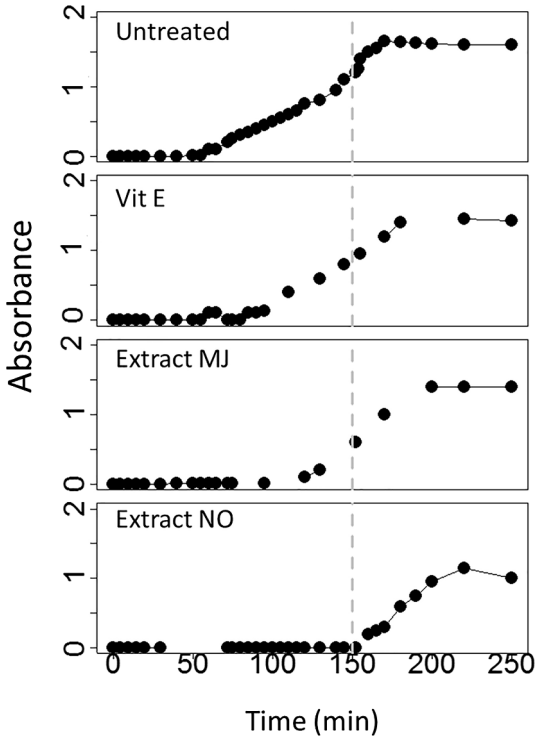

Fig. 4. Kinetics curves of lipid oxidation for: (A) Untreated LDLs. (B) LDLs treated with extracts MJ. (C) LDLs treated with extracts NO from *Hypnea musciformis* and (D) LDLs treated with vitamin E. Phase 1. Protection period or lag or latency phase (Lag phase). Phase 2. Propagation phase (large quantities of unsaturated fatty acids are formed). Phase 3. Decay of all antioxidants present in the LDLs. LDLs treated with extracts of *H. musciformis* had a much longer lag phase indicating a protective role of these extracts against lipid oxidation. The X axis is the same for the four graphs in this figure.

The optical rotation of compound **1** was $[\alpha]^{20} = -53.4$, which allows us to conclude that compound 1 is (−)-epicatechin.

Our results agree with those reported by researchers (Mohamed, Hashim, & Rahman, 2012; Rioux, Beaulieu, & Turgeon, 2017; Santoso, Yoshie, & Suzuki, 2002) who previously isolated catechins and flavonoids in several species of brown, green and red algae. Other authors have also reported catechins in red algae (Yoshie, Wang, Petillo, & Suzuki, 2000) such as *Porphyra,* in which they have found other

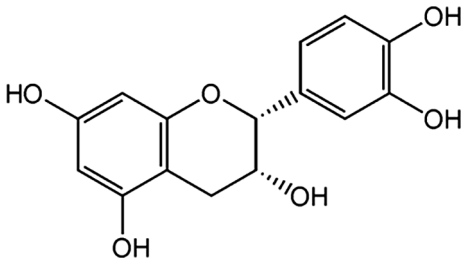

Fig. 5. Chemical structure of compound **1**, (−) – epicatechin.

antioxidants such as rutin, morin, hesperidin, caffeic acid, catechin, catechol and epigallocatechin gallate (Fernando, Nah, & Jeon, 2016; Noel & Yang, 2001).

Catechins in *H. musciformis* may be responsible for inhibiting the oxidation of LDLs with greater antioxidant activity than vitamin E (Fitó et al., 2000). Hence, we attribute the antioxidant activity of extract MJ to the presence of (−)-epicatechin, although the presence of other substances contributing to antioxidant activity, such as carotenes or other pigments, cannot be ruled out (Agatonovic-Kustrin et al., 2018; Grzesik, Naparło, Bartosz, & Sadowska-Bartosz, 2018).

The structure corresponding to compound **2**, from extract NO, is presented in Fig. 6 (dataset Rozo et al., 2019) and agrees with the chemical structure of a dihydrochalcone known as phloretin (Richard et al., 2013; Sun, Li, & Liu, 2015). This phenolic compound has been reported to be responsible for antioxidant activity in brown algae (Jiménez-Escrig, Jiménez-Jiménez, Pulido, & Saura-Calixto, 2001; Peng et al., 2015; Rezk, Haenen, Van der Vijgh, & Bast, 2002).

The antioxidant properties of phloretin in LDLs have been determined, and its protective effect against lipid peroxidation is promising, even in hepatic cells (Nithiya & Udayakumar, 2016; Zuo et al., 2014).

The TPC and antioxidant activity of extract NO assessed through the ABTS and TBARS assays (Table 3) may be partly explained by the presence of phloretin in the extract (Rezk et al., 2002). However, it is possible that other molecules present in extract NO could increase antioxidant activity in a synergistic manner.

The results of the TBARS assays with *Perna viridis* and the presence of phloretin in extracts of the red alga *H. musciformis* lead us to propose these extracts as an alternative to synthetic antioxidants used to preserve fish products (Maqsood et al., 2013) and other foodstuffs.

Red algae are an important source of active compounds in comparison to other algal groups (El Gamal, 2010). The exposure of algae to

Table 6
Latency phase duration for extracts of *Hypnea musciformis* as protectors against LDL oxidation.

	Untreated LDLs	LDLs treated with extract MJ	LDLs treated with extract NO	LDLs treated with vitamin E
Time min ± sd	72 ± 4.24	136.3 ± 1.7	156.0 ± 16.73	88.6 ± 10.9

Data are expressed as the means ± standard deviations (sd), (n = 5).

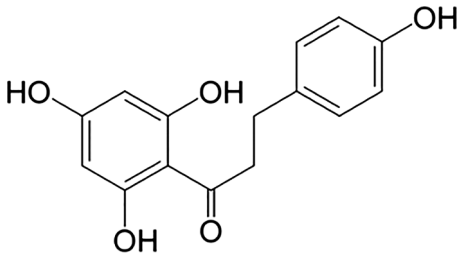

Fig. 6. Chemical structure of compound **2**, phloretin.

the particular environmental conditions of the ocean may cause metabolic shifts resulting in permanent changes in their phenol content (Stengel et al., 2011). Ocean acidification, for instance, appears to have a deleterious effect on catechin concentrations in algae (Kumar et al., 2018). The long geological history of the Santa Marta coast, the complexity of marine habitats along that coast and the influence of seasonal local upwelling (Delgado, Palacio, & Aguirre, 2008) may partly explain the high concentrations of polyphenols in *H. musciformis* from that area.

4. Conclusions

Extraction with supercritical fluid (SFE) can be optimized to obtain a large amount of extract from algal samples by using low pressure and a high concentration of ethanol in the solvent (8% EtOH with CO_2).

We have shown that *H. musciformis* extracts contain polyphenols. Since these compounds can interact with the proteins involved in regulating the glycemic index, our results provide a foundation for future research to evaluate the consumption of *H. musciformis* as a functional food with benefits for the treatment and/or prevention of diseases such as diabetes and obesity. The potential of the algal extracts to prevent LDL oxidation was between 42 and 62% higher than the protection granted by vitamin E, which is a natural antioxidant of LDLs in the human body. Our results of measuring LDL peroxidation *in vitro* are expected to be consistent with the LDL environment inside the human body. Therefore, we consider that the consumption of *H. musciformis* by local populations in the Caribbean is a positive habit and could potentially help to prevent the onset of diseases such as atherosclerosis in healthy individuals.

The presence of the antioxidant compounds phloretin and (−)-epicatechin in extracts of *H. musciformis* provides evidence of the high potential of these extracts as functional foods. Caribbean red algae can be regarded as a good source of antioxidants with a large number of applications that benefit human well-being.

Ethics statements

Statement in the manuscript: that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

This work was funded by Universidad Jorge Tadeo Lozano (Internal Grant 548-10-13). Algal samples were collected under Collecting Permit “Permiso Marco de Colecta IDB 0401” (Resolución 1271 del 23 de octubre de 2014, Ministerio de Medio Ambiente y Desarrollo Sostenible (Colombian Ministry of the Environment and Sustainable Development). The authors acknowledge the efforts of Ramón Alejandro Plazas, who collected and identified the algae used in this study. Professor Ligia Rodriguez helped us design the LDL extraction methods. This work is part of the requirements of the first author (Gladys Rozo) for obtaining the degree of Doctor in Marine Sciences from Universidad Jorge Tadeo Lozano.

References

Agatonovic-Kustrin, S., Kustrin, E., Angove, M. J., & Morton, D. W. (2018). A screening method for cardiovascular active compounds in marine algae. *Journal of Chromatography A, 1550*, 57–62. https://doi.org/10.1016/j.chroma.2018.03.054.

Ananingsih, V. K., Sharma, A., & Zhou, W. (2013). Green tea catechins during food processing and storage: A review on stability and detection. *Food Research International, 50*(2), 469–479. https://doi.org/10.1016/j.foodres.2011.03.004.

Andrade, P. B., Barbosa, M., Matos, R. P., Lopes, G., Vinholes, J., Mouga, T., & Valentão, P. (2013). Valuable compounds in macroalgae extracts. *Food Chemistry, 138*(2–3), 1819–1828. https://doi.org/10.1016/j.foodchem.2012.11.081.

Antolovich, M., Prenzler, P. D., Patsalides, E., McDonald, S., & Robards, K. (2002). Methods for testing antioxidant activity. *The Analyst, 127*(1), 183–198. https://doi.org/10.1039/b009171p.

Asep, E. K., Jinap, S., Jahurul, M. H. A., Zaidul, I. S. M., & Singh, H. (2013). Effects of polar cosolvents on cocoa butter extraction using supercritical carbon dioxide. *Innovative Food Science and Emerging Technologies, 20*, 152–160. https://doi.org/10.1016/j.ifset.2013.06.010.

Asep, E. K., Jinap, S., Tan, T. J., Russly, A. R., Harcharan, S., & Nazimah, S. A. H. (2008). The effects of particle size, fermentation and roasting of cocoa nibs on supercritical fluid extraction of cocoa butter. *Journal of Food Engineering, 85*(3), 450–458. https://doi.org/10.1016/j.jfoodeng.2007.08.008.

Balamurugan, M., & Selvam, G. G. (2013). Biochemical study and GC-MS analysis of Hypnea musciformis (Wulf.) Lamouroux. *American-Eurasian Journal of Scientific Research, 8*(3), 117–123. https://doi.org/10.5829/idosi.aejsr.2013.8.3.12071.

Batista, A. E., Charles, M., Mancini-filho, J., & Vidal, A. (2009). Las algas marinas como fuentes de fitofármacos antioxidantes Seaweeds as sources of antioxidant phytomedicines. *Revista Cubana de Plantas Medicinales, 14*(2), 1–18 https://doi.org/1028-4796.

Bayod, C., Villarroel, M. T., Peréz Lorenz, J. B., & Puzo, J. (2013). Arteriosclerosis. Factores de riesgo cardiovascular. *Medicine (Spain), 11*(40), 2283–2295. https://doi.org/10.1016/S0304-5412(13)70635-8.

Benelli, P., Riehl, C. A. S., Smânia, A., Smânia, E. F. A., & Ferreira, S. R. S. (2010). Bioactive extracts of orange (Citrus sinensis L. Osbeck) pomace obtained by SFE and low pressure techniques: Mathematical modeling and extract composition. *Journal of Supercritical Fluids, 55*(1), 132–141. https://doi.org/10.1016/j.supflu.2010.08.015.

Blunt, J. W., Copp, B. R., Hu, W. P., Munro, M. H., Northcote, P. T., & Prinsep, M. R. (2009). Marine natural products. *Natural Product Reports, 26*(2), 170–244. https://doi.org/10.1039/b805113p.

Cardozo, K. H. M., Guaratini, T., Barros, M. P., Falcão, V. R., Tonon, A. P., Lopes, N. P., & Pinto, E. (2007). Metabolites from algae with economical impact. *Comparative Biochemistry and Physiology – C Toxicology and Pharmacology, 146*(1-2 SPEC. ISS.), 60–78. https://doi.org/10.1016/j.cbpc.2006.05.007.

Chakraborty, K., Joseph, D., & Praveen, N. K. (2013a). Antioxidant activities and phenolic contents of three red seaweeds (Division: Rhodophyta) harvested from the Gulf of Mannar of Peninsular India. *Journal of Food Science and Technology, 1–12*. https://doi.org/10.1007/s13197-013-1189-2.

Chakraborty, K., Praveen, N. K., Vijayan, K. K., & Rao, G. S. (2013b). Evaluation of phenolic contents and antioxidant activities of brown seaweeds belonging to *Turbinaria* spp. (Phaeophyta, Sargassaceae) collected from Gulf of Mannar. *Asian Pacific Journal of Tropical Biomedicine, 3*(1), 8–16. https://doi.org/10.1016/S2221-1691(13)60016-7.

Combs, G. F. (2008). *The vitamins* (3rd ed.). United States of America: E. A. Press.

D'Orazio, N., Gemello, E., Gammone, M. A., De Girolamo, M., Ficoneri, C., & Riccioni, G. (2012). Fucoxantin: A treasure from the sea. *Marine Drugs, 10*(3), 604–616. https://doi.org/10.3390/md10030604.

Darias-Jerez, J. (2016). La Biodiversidad de las Algas Marinas como fuente de interés farmacológico. *Revista de Medio Ambiente, 9*, 1–7.

Dawes, C. J., & Mathieson, A. C. (2008). *The seaweeds of Florida.* Gainesville, USA: University Press of Florida591.

de Almeida, C. L. F., Falcão, H.deS., Lima, G. R.d. M., Montenegro, C.deA., Lira, N. S., de Athayde-Filho, P. F., ... Batista, L. M. (2011). Bioactivities from marine algae of the genus *Gracilaria*. *International Journal of Molecular Sciences, 12*(7), 4550–4573. https://doi.org/10.3390/ijms12074550.

Delgado, C. M., Palacio, A. J., & Aguirre, J. N. (2008). Variación vertical y estacional de la comunidad de macroalgas. *Gestión y Ambiente, 11*(3), 27–42. http://www.bdigital.unal.edu.co/18244/1/14018-41513-1-PB.pdf.

Domínguez, H. (2013). Algae as a source of biologically active ingredients for the formulation of functional foods and nutraceuticals. *Functional Ingredients from Algae for Foods and Nutraceuticals,* 1–19. https://doi.org/10.1533/9780857098689.1.

Echavarría, B. Z., Franco, A. S., & Martínez, A. M. (2009). Evaluación de la actividad antioxidante y determinación del contenido de compuestos fenólicos en extractos de macroalgas del Caribe Colombiano. *Vitae, 16*(1), 126–131. https://doi.org/10.1016/j.aprim.2011.07.010.

El Gamal, A. A. (2010). Biological importance of marine algae. *Saudi Pharmaceutical Journal, 18*(1), 1–25. https://doi.org/10.1016/j.jsps.2009.12.001.

Esterbauer, H., & Striegl, G. (1989). Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Biology and Medicine, 6*(I), 67–75. https://doi.org/10.3109/10715768909073429.

Fernando, I. P. S., Nah, J. W., & Jeon, Y. J. (2016). Potential anti-inflammatory natural

products from marine algae. *Environmental Toxicology and Pharmacology, 48*, 22–30. https://doi.org/10.1016/j.etap.2016.09.023.
Fishbein, M. C., & Fishbein, G. A. (2015). Arteriosclerosis: Facts and fancy. *Cardiovascular Pathology, 24*(6), 335–342. https://doi.org/10.1016/j.carpath.2015.07.007.
Fitó, M., Isabel Covas, M., Lamuela-Raventós, R., Vila, J., de la Torre, C., & Marrugat, J. (2000). Aceite de oliva e inhibición de la oxidación de las lipoproteínas de baja densidad. Importancia de los compuestos fenólicos. *Medicina Clínica, 115*(5), 166–169. https://doi.org/10.1016/S0025-7753(00)71497-7.
Gordon, M. H. (2001). Measuring antioxidant activity. In J. Pokorny, N. Yanishlieva, & M. H. Gordon (Eds.). *Antioxidants in food: Practical applications*. CRC Press.
Grzesik, M., Naparło, K., Bartosz, G., & Sadowska-Bartosz, I. (2018). Antioxidant properties of catechins: Comparison with other antioxidants. *Food Chemistry, 241*(August 2017), 480–492. https://doi.org/10.1016/j.foodchem.2017.08.117.
Guedes, É.a. C., da Silva, T. G., Aguiar, J. S., de Barros, L. D., Pinotti, L. M., & Sant'Ana, A. E. G. (2013). Cytotoxic activity of marine algae against cancerous cells. *Revista Brasileira de Farmacognosia, 23*(4), 668–673. https://doi.org/10.1590/S0102-695X2013005000060.
Gutiérrez-Rodríguez, A. G., Juárez-Portilla, C., Olivares-Bañuelos, T., & Zepeda, R. C. (2018). Anticancer activity of seaweeds. *Drug Discovery Today, 23*(2), 434–447. https://doi.org/10.1016/j.drudis.2017.10.019.
Hernández-Leiva, E. (2011). Epidemiología del síndrome coronario agudo y la insuficiencia cardiaca en Latinoamérica. *Revista Espanola de Cardiologia, 64*(SUPPL. 2), 34–43. https://doi.org/10.1016/j.recesp.2011.01.022.
Hostettmann, K., Gupta, M. P., Marston, A., & Ferreira Quiróz, E. (2008). Handbook of Strategies for the Isolation of Bioactive Natural Product. *Convenio Andrés Bello, Serie de Ciencia y Tecnología, 160*, 114 I.S.B.N.: 978–958-698-210-8.
Hulthe, J., & Fagerberg, B. (2002). Circulating oxidized LDL is associated with subclinical atherosclerosis development and inflammatory cytokines (AIR study). *Arteriosclerosis, Thrombosis, and Vascular Biology, 22*(7), 1162–1167. https://doi.org/10.1161/01.ATV.0000021150.63480.CD.
Ibrahim, M., Salman, M., Kamal, S., Rehman, S., Razzaq, A., & Akash, S. H. (2017). *Algae-based biologically active compounds. Algae based polymers, blends, and composites: chemistry, biotechnology and materials science.* Elsevier Inc.https://doi.org/10.1016/B978-0-12-812360-7.00006-9.
Jiménez-Escrig, A., Jiménez-Jiménez, I., Pulido, R., & Saura-Calixto, F. (2001). Antioxidant activity of fresh and processed edible seaweeds. *Journal of the Science of Food and Agriculture, 81*(5), 530–534. https://doi.org/10.1002/jsfa.842.
Kumar, A., AbdElgawad, H., Castellano, I., Selim, S., Beemster, G. T. S., Asard, H., ... Palumbo, A. (2018). Effects of ocean acidification on the levels of primary and secondary metabolites in the brown macroalga Sargassum vulgare at different time scales. *Science of The Total Environment, 643*, 946–956. https://doi.org/10.1016/j.scitotenv.2018.06.176.
Kumar, R., Tayade, A., Chaurasia, O., Sunil, H., & Singh, S. B. (2010). Evaluation of antioxidant activities and total phenol and flavonoid content of the hydro-alcoholic extracts of Rhodiola sp. *Pharmacognosy Journal, 2*(11), 431–435. https://doi.org/10.1016/S0975-3575(10)80027-6.
Laguerre, M., Lecomte, J., & Villeneuve, P. (2007). Evaluation of the ability of antioxidants to counteract lipid oxidation: Existing methods, new trends and challenges. *Progress in Lipid Research, 46*(5), 244–282. https://doi.org/10.1016/j.plipres.2007.05.002.
Lee, J. C., Hou, M. F., Huang, H. W., Chang, F. R., Yeh, C. C., Tang, J. Y., & Chang, H. W. (2013). Marine algal natural products with anti-oxidative, anti-inflammatory, and anti-cancer properties. *Cancer Cell International, 13*(1), 1. https://doi.org/10.1186/1475-2867-13-55.
Li, S., Chen, G., Zhang, C., Wu, M., Wu, S., & Liu, Q. (2014). Research progress of natural antioxidants in foods for the treatment of diseases. *Food Science and Human Wellness, 3*(3–4), 110–116. https://doi.org/10.1016/j.fshw.2014.11.002.
Littler, D. S., Littler, M. M., Bucher, K. E., & Norris, J. N. (1989). *Marine plants of the Caribbean. A field guide from Florida to Brazil.* Washington D C: Smithsonian Institution Press251.
Lönn, M. E., Dennis, J. M., & Stocker, R. (2012). Actions of antioxidants in the protection against atherosclerosis. *Free Radical Biology and Medicine, 53*(4), 863–884. https://doi.org/10.1016/j.freeradbiomed.2012.05.027.
Lordan, S., Ross, R. P., & Stanton, C. (2011). Marine bioactives as functional food ingredients: Potential to reduce the incidence of chronic diseases. *Marine Drugs, 9*(6), 1056–1100. https://doi.org/10.3390/md9061056.
Maiorino, M., Zamburlini, A., Roveri, A., & Ursini, F. (1995). Copper-induced lipid peroxidation in liposomes, micelles, and LDL: Which is the role of vitamin E? *Free Radical Biology and Medicine, 18*(1), 67–74. https://doi.org/10.1016/0891-5849(94)00103-Q.
Maqsood, S., Benjakul, S., & Shahidi, F. (2013). Emerging role of phenolic compounds as natural food additives in fish and fish products. *Critical Reviews in Food Science and Nutrition, 53*(2), 162–179. https://doi.org/10.1080/10408398.2010.518775.
Mendiola, J. A., Herrero, M., Castro, P. M., & Ibáñez, E. (2013). Supercritical fluid extraction. In M. A. Rostagno, & J. M. Prado (Eds.). *Natural product extraction: principles and applications* (pp. 196–225). Royal Society of Chemistry.
Mendivil, C. O., Sierra, I. D., & Pérez, C. E. (2004). Valoración del riesgo cardiovascular global y prevalencia de dislipemias según los criterios del NCEP-ATP III en una población adulta de Bogotá, Colombia. *Clínica e Investigación En Arteriosclerosis, 16*(3), 99–107. https://doi.org/10.1016/S0214-9168(04)78970-6.
Mirończuk-Chodakowska, I., Witkowska, A. M., & Zujko, M. E. (2018). Endogenous non-enzymatic antioxidants in the human body. *Advances in Medical Sciences, 63*(1), 68–78. https://doi.org/10.1016/j.advms.2017.05.005.
Mohamed, S., Hashim, S. N., & Rahman, H. A. (2012). Seaweeds: A sustainable functional food for complementary and alternative therapy. *Trends in Food Science and Technology, 23*(2), 83–96. https://doi.org/10.1016/j.tifs.2011.09.001.
Murcia, J. Luis, & Alanis, G. (2003). Alimentos funcionales. *Distribución y Consumo, 5*, 48–50.
Nagai, T., & Yukimoto, T. (2003). Preparation and functional properties of beverages made from sea algae. *Food Chemistry, 81*(3), 327–332. https://doi.org/10.1016/S0308-8146(02)00426-0.
Nithiya, T., & Udayakumar, R. (2016). In vitro antioxidant properties of phloretin—An important phytocompound. *Journal of Biosciences and Medicines, 4*(January), 85–94.
Noel, L., & Yang, S. B. (2001). Flavonoids from the Red Alga Acanthophora spicifera. *Chinese Journal of Chemistry, 19*(29932030), 1097. https://doi.org/10.1002/cjoc.20010191116.
Nohynek, G. J., Antignac, E., Re, T., & Toutain, H. (2010). Safety assessment of personal care products/cosmetics and their ingredients. *Toxicology and Applied Pharmacology, 243*(2), 239–259. https://doi.org/10.1016/j.taap.2009.12.001.
Pangestuti, R., & Kim, S. K. (2011). Biological activities and health benefit effects of natural pigments derived from marine algae. *Journal of Functional Foods, 3*(4), 255–266. https://doi.org/10.1016/j.jff.2011.07.001.
Peng, Y., Hu, J., Yang, B., Lin, X. P., Zhou, X. F., Yang, X. W., & Liu, Y. (2015). *Chemical composition of seaweeds. Seaweed sustainability: Food and non-food.* Elsevier Inc.https://doi.org/10.1016/B978-0-12-418697-2.00005-2.
Pereira, C. G., & Meireles, M. A. (2010). Supercritical fluid extraction of bioactive compounds: Fundamentals, applications and economic perspectives. *Food and Bioprocess Technology, 3*(3), 340–372. https://doi.org/10.1007/s11947-009-0263-2.
Perez, Y. (2007). Oxidación de las LDL (lipoproteínas de baja densidad) y su relación con la patogénesis de la aterosclerosis. *Revista de Ciencias Biologicas, 38*(1), 3–11. https://doi.org/10.1016/S0214-9168(02)78822-0.
R Core Team (2016). *R: A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing.
Radulovich, R., Umanzor, S., & Cabrera, R. (2013). *Algas Tropicales Cultivo y Uso como Alimento 2013 (Tropical seaweeds: cultivation and use as food), Vol. 2013*. San josé de Costa Rica: U. de C. Rica.
Radulovich, R., Neori, A., Valderrama, D., Reddy, C. R. K., Cronin, H., & Forster, J. (2015). *Farming of seaweeds. Seaweed sustainability: Food and non-food applications.* Elsevier Inc.https://doi.org/10.1016/B978-0-12-418697-2.00003-9.
Re, R., PellegriniI, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved abts radical. *Free Radical Biology & Medicine, 26*(9–10), 1231–1237.
Rezk, B. M., Haenen, G. R. M. M., Van der Vijgh, W. J. F., & Bast, A. (2002). The antioxidant activity of phloretin: The disclosure of a new antioxidant pharmacophore in flavonoids. *Biochemical and Biophysical Research Communications, 295*(1), 9–13. https://doi.org/10.1016/S0006-291X(02)00618-6.
Rice-Evans, C., Miller, N., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science, 2*(4), 152–159. https://doi.org/10.1016/S1360-1385(97)01018-2.
Richard, T., Temsamani, H., Cantos-Villar, E., & Monti, J. P. (2013). *Application of LC-MS and LC-NMR techniques for secondary metabolite identification. Advances in botanical research, Vol. 67 1st ed.*Elsevier Ltd.https://doi.org/10.1016/B978-0-12-397922-3.00002-2.
Rioux, L. E., Beaulieu, L., & Turgeon, S. L. (2017). Seaweeds: A traditional ingredients for new gastronomic sensation. *Food Hydrocolloids, 68*, 255–265. https://doi.org/10.1016/j.foodhyd.2017.02.005.
Rozo, G., Rozo, C., Puyana, M., Ramos, F. A., Almonacid, C., & Castro, H. (2019). H1NMR data of chemical structure of compounds. *Mendeley Data, v1* (DOI is reserved).
Sakamoto, S., Hatakeyama, M., Ito, T., & Handa, H. (2012). Tools and methodologies capable of isolating and identifying a target molecule for a bioactive compound. *Bioorganic and Medicinal Chemistry, 20*(6), 1990–2001. https://doi.org/10.1016/j.bmc.2011.12.022.
Salvayre, R., Negre-Salvayre, A., & Camaré, C. (2016). Oxidative theory of atherosclerosis and antioxidants. *Biochimie, 125*, 281–296. https://doi.org/10.1016/j.biochi.2015.12.014.
Sánchez-Muniz, F. J., Bocanegra de Juana, A., Bastida, S., & Benedí, J. (2013). Algae and cardiovascular health. *Functional Ingredients from Algae for Foods and Nutraceuticals*. https://doi.org/10.1533/9780857098689.2.369.
Santoso, J., Yoshie, Y., & Suzuki, T. (2002). The distribution and profile of nutrients and catechins of some Indonesian seaweeds. *Fisheries Science, 68*, 1647–1648. https://doi.org/10.2331/FISHSCI.68.SUP2_1647.
Sarojini, Y., Sujatha, B., & Santha Rao, P. (2016). The variation in distribution of total phenols and antioxidant activity in five species of marine macro algae. *Der Pharmacia Lettre, 8*(1), 30–37.
Shahidi, F., & Zhong, Y. (2005). Lipid oxidation: Measurement methods. *Bailey's Industrial Oil and Fat Products, 3*, 357–385. https://doi.org/10.1002/047167849X.bio050.
Shahidi, F., & Zhong, Y. (2015). Measurement of antioxidant activity. *Journal of Functional Foods, 18*, 757–781. https://doi.org/10.1016/j.jff.2015.01.047.
Siriwardhana, N., Kim, K. N., Lee, K. W., Kim, S. H., Ha, J. H., Song, C. B., ... Jeon, Y. J. (2008). Optimisation of hydrophilic antioxidant extraction from Hizikiafusiformis by integrating treatments of enzymes, heat and pH control. *International Journal of Food Science and Technology, 43*(4), 587–596. https://doi.org/10.1111/j.1365-2621.2006.01485.x.
Siti, H. N., Kamisah, Y., & Kamsiah, J. (2015). The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease (a review). *Vascular Pharmacology, 71*, 40–56. https://doi.org/10.1016/j.vph.2015.03.005.
Sonani, R. R., Rastogi, R. P., & Madamwar, D. (2017). *Natural antioxidants from algae: A therapeutic perspective. Algal green chemistry: Recent progress in biotechnology.* Elsevier B.Vhttps://doi.org/10.1016/B978-0-444-63784-0.00005-9.
Stengel, D. B., Connan, S., & Popper, Z. A. (2011). Algal chemodiversity and bioactivity: Sources of natural variability and implications for commercial application. *Biotechnology Advances, 29*(5), 483–501. https://doi.org/10.1016/j.biotechadv.2011.

05.016.
Sun, Y., Li, W., & Liu, Z. (2015). Preparative isolation, quantification and antioxidant activity of dihydrochalcones from Sweet Tea (Lithocarpus polystachyus Rehd.). *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences, 1002*, 372–378. https://doi.org/10.1016/j.jchromb.2015.08.045.
Thomas, N. V., & Kim, S. K. (2011). Potential pharmacological applications of polyphenolic derivatives from marine brown algae. *Environmental Toxicology and Pharmacology, 32*(3), 325–335. https://doi.org/10.1016/j.etap.2011.09.004.
Tsikas, D. (2017). Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. *Analytical Biochemistry, 524*, 13–30. https://doi.org/10.1016/j.ab.2016.10.021.
Villalpando, D. C., Alvarez, C., & Gómez, A. (2017). Advanced oxidation protein products and their relationship with cardiovascular risk factors in young apparently healthy people. *Clínica e Investigación En Arteriosclerosis (English Edition), 29*(5), 209–215. https://doi.org/10.1016/j.artere.2017.04.001.
Wijesekara, I., Pangestuti, R., & Kim, S. K. (2011). Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. *Carbohydrate Polymers, 84*(1), 14–21. https://doi.org/10.1016/j.carbpol.2010.10.062.
Winklhofer-Roob, B. M., Faustmann, G., & Roob, J. M. (2017). Low-density lipoprotein oxidation biomarkers in human health and disease and effects of bioactive compounds. *Free Radical Biology and Medicine, 111*(April), 38–86. https://doi.org/10.1016/j.freeradbiomed.2017.04.345.
Witztum, J. L., & Hörkkö, S. (1997). The role of oxidized LDL in atherogenesis: Immunological response and anti-phospholipid antibodies. *Annals of the New York Academy of Sciences, 811*, 88–99. https://doi.org/10.1111/j.1749-6632.1997.tb51992.x.
Yildiz, G., Vatan, Ö., Çelikler, S., & Dere, Ş. (2011). Determination of the phenolic compounds and antioxidative capacity in Red Algae Gracilaria bursa-pastoris. *International Journal of Food Properties, 14*(3), 496–502. https://doi.org/10.1080/10942910903256949.
Yoshida, H., & Kisugi, R. (2010). Mechanisms of LDL oxidation. *Clinica Chimica Acta, 411*(23–24), 1875–1882. https://doi.org/10.1016/j.cca.2010.08.038.
Yoshie, Y., Wang, W., Petillo, D., & Suzuki, T. (2000). Distribution of catechins in Japanese seaweeds. *Fisheries Science, 66*(5), 998–1000. https://doi.org/10.1046/j.1444-2906.2000.00160.x.
Zuo, A. R., Yu, Y. Y., Shu, Q. L., Zheng, L. X., Wang, X. M., Peng, S. H., ... Cao, S. W. (2014). Hepatoprotective effects and antioxidant, antityrosinase activities of phloretin and phloretin isonicotinyl hydrazone. *Journal of the Chinese Medical Association, 77*(6), 290–301. https://doi.org/10.1016/j.jcma.2014.01.007.