Characterization and *in vitro* evaluation of seaweed species as potential functional ingredients to ameliorate metabolic syndrome

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ABSTRACT

This study aimed at identifying seaweed species with optimal characteristics to develop multifunctional foods for metabolic syndrome (MetS) management. Mass spectrometry chemical characterization and bioactive profile evaluation of methanolic extracts from seven commonly consumed seaweeds were compared. Monomeric sugars namely mannitol, fucitol, xylitol and their sulphated analogs as well as lipids (phosphatidic acid, octadecenoic acid, and prostaglandin 2 α) were detected in seaweeds. *Himanthalia elongata* showed the highest phenolic content (24.04 μ mol GAE/g), and antioxidant activity. This species was the only one showing angiotensin converting enzyme-I inhibitory activity (IC₅₀=65 μ g/mL). *U. pinnatifida* and *H. elongata* extracts were notably more effective reducing pro-inflammatory molecules in lipopolysaccharide-induced RAW 264.7 macrophages. Finally, *Ulva* spp., *Palmaria palmata*, *U. pinnatifida* and *H. elongata* significantly inhibited triglyceride accumulation in mature 3T3-L1 adipocytes (43-52% inhibition). Among seaweeds, *H. elongata* showed the highest potential to be used as ingredient in the development of new functional foods for MetS management.

Keywords: seaweed, antioxidant; anti-hypertensive; anti-inflammatory; triglyceride accumulation, metabolic syndrome

1. Introduction

The modern lifestyle is leading to changes in the eating patterns of global population towards diets rich in refined sugars, salt, saturated fats and energy-dense foods (Hyseni et al., 2017). These modern diets contribute to the development of chronic diseases responsible for 70% of all deaths worldwide (WHO, 2017). Metabolic syndrome (MetS) is becoming one of the most important health concerns in the last years, due to its rising prevalence (Grundy, 2016). This syndrome encompasses a constellation of several risk factors including hyperglycemia, raised blood pressure, dyslipidemia and visceral obesity that predispose to the development of cardiovascular disease and type 2 diabetes mellitus (Alberti et al., 2009). According to the National Cholesterol Education Program Adult treatment Panel III (NCEP ATP III, 2002) definition, MetS is present if three or more of the following five criteria are met: waist circumference over 102 cm (men) or 88 cm (women), blood pressure over 130/85 mmHg, fasting triglyceride level over 150 mg/dl, fasting high-density lipoprotein (HDL) cholesterol level less than 40 mg/dl (men) or 50 mg/dl (women) and fasting blood sugar over 100 mg/dl. Indeed, visceral obesity and insulin resistance are key etiological factors of MetS development. Obesity causes an increased concentration of free fatty acids (FFA) that may cause insulin resistance, oxidative stress and inflammation (Dandona et al., 2005). The search for efficient and economic strategies for prevention of MetS is crucial to ameliorate its impact on global health and economy. Healthy diets may play a key role in reducing the incidence of MetS (Grosso et al., 2017). In this context, many studies have provided evidence on the role of foods rich in bioactive compounds to counteract the different components of MetS.

Due to the current consumers trend to embrace organically grown, natural and healthy foods from clean environments, edible macroalgae (seaweed) are receiving an increasing acceptance. Edible seaweeds have been consumed as food and traditional medicines since ancient times in Asian countries such as Japan, Korea and China (Levine, 2016). Seaweed

consumption in western societies is lower than in Asia, but they have been traditional consumed in countries such as England, Ireland, Scotland, Scandinavia, and Canada for human and domestic animal feeding (Delaney et al., 2016). The nutritional and beneficial health effects derived from seaweed consumption has stimulated their inclusion as functional ingredients in a broad range of foodstuffs (sushi, pasta, biscuits, bread and beverages, among others), and can be also consumed fresh or dried as whole foods (Rioux, Beaulieu and Turgeon, 2017). Moreover, seaweeds are commonly used in the production of gelling agents such as alginate or agar by the food industry in western countries (Smit 2004). The inclusion of seaweed extracts enriched in different bioactive compounds as powdered ingredients could represent a promising tool for the design of multifunctional foods or nutraceuticals.

Among the eight most important seaweeds used for human consumption are species from *Porphyra* spp. (Nori), *Laminaria japonica* (Kombu), *Undaria pinnatifida* (Wakame), *Ulva* spp. (Sea Lettuce), *Palmaria palmata* (Dulse) and *Chondrus crispus* (Irish moss) (Hassan, 2009). They are attracting growing interest nowadays as excellent sources of essential fatty acids with a nutritionally ideal n-6/n-3 fatty acid ratio, polysaccharides, with relatively high protein content, including all the essential amino acids, minerals, vitamins and trace elements. In addition, several *in vivo* studies support the positive effect of seaweed products when consumed directly or as dietary supplements on health by reducing body weight (Okada et al., 2011), lipid oxidation (Grasa-López et al., 2016), oxidative stress and insulin resistance (Tong et al., 2015). Seaweed extracts have also shown antimicrobial (Cox, Abu-Ghannam, & Gupta, 2010), anti-inflammatory (Han et al., 2015), anti-diabetic (Kang et al., 2013) and prebiotic effects (Charoensiddhi et al., 2017). All these effects have been attributed to the presence of bioactive compounds such as polysaccharides, bioactive lipids, trace elements and phenolic compounds (Gupta & Abu-Ghannam, 2011).

All these characteristics suggest a promising potential to develop novel seaweed products with commercial interest for functional food applications to enhance human health (Murray, Dordevic, Ryan, & Bonham, 2017). For this purpose, consideration must be given to the selection of seaweed species with the best phytochemical and bioactivity profile. Different algal species contain varying combinations and concentrations of bioactive compounds and bioactive properties (Murray et al., 2017). To date there is no research that has examined and compared the potential of different seaweeds with the aim to develop multifunctional food products for amelioration of MetS progression. Therefore, the aim of the present study was to chemically characterize seven seaweed species and to evaluate their potential for *in vitro* antihypertensive, anti-inflammatory, antioxidant and triacylglyceride-lowering activities.

2. Materials and methods

2.1. Chemicals

Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS) from *Escherichia coli* O5:B55, Folin-Ciocalteu phenol reagent, Trolox, 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein, 2,2'-azobis (2-methyl-propionamidine dihydrochloride) (AAPH), captopril and 3,4,5-dimethylthiazol-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin (5,000 U/mL), and phosphate buffer saline (PBS) were purchased from Lonza (Cultek S.L.U, Madrid, Spain). Fetal bovine serum (FBS) of South American origin was obtained from Fisher Scientific (Madrid, Spain). Cell culture flasks and plates were obtained from Sarstedt (Nümbrecht, Germany).

2.2. Sample material

Seaweed species *Porphyra spp.* (Nori), *Undaria pinnatifida* (Wakame), *Ulva spp.* (Sea lettuce), *Himanthalia elongata* (Sea thong), *Chondrus crispus* (Irish moss), *Laminaria*

ochroleuca (Kombu), and *Palmaria palmata* (Dulse) were collected and provided by a local producer (Portomuiños; Galicia, Spain). Fresh raw materials were lyophilized and powdered using a grinding mill and kept at -80°C until further analysis.

2.3. Preparation of methanolic extracts

Seaweed extraction was carried out using 1g of powdered sample and homogenized in 2 x 10 mL of 50% aqueous methanol by magnetic stirring for 1 h. The two extracts were centrifuged at 1,635 x g for 10 min at 4 °C. Supernatants were combined and filtered using Whatman Grade 1 filter paper. Filtrates were evaporated under reduced pressure (SpeedVac Concentrator, Thermo Fisher Scientific, Madrid, Spain) to remove solvent and held at -80°C until analysis. For all cell assays, samples were reconstituted in free-serum cell culture media to the appropriate concentration for testing.

2.4 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS analysis of the seaweed extracts were performed on a Q-ToF Premier mass spectrometer (Waters Corporation, Milford, MA, USA) coupled to an Alliance 2695 HPLC system (Waters Corporation, Milford, MA, USA). Separation of compounds was achieved on an Atlantis T3 C18 column (Waters Corporation, Milford, USA, 100 mm x 2.1 mm; 3 µm particle size) using 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Column temperature was maintained at 40 °C. A stepwise gradient from 10% to 90% solvent B was applied at a flow rate of 0.3 mL/min for 18 min. Electrospray mass spectra data recorded on a negative ionisation mode for a mass range m/z 100 to m/z 1000. Capillary voltage and cone voltage were set at 3 kV and 30 V, respectively. Collision induced fragmentation (CID) of the analytes was achieved using 12 eV to 20 eV energy with argon as the collision gas.

2.5. Total phenolic content

Folin-Ciocalteu method (Slinkard, 1977) was used with slight modifications. A volume of 140 μ L of the sample extract was mixed with 280 μ L of Folin-Ciocalteu reagent previously diluted (1:10, v/v) and 980 μ L of 42.86 mM sodium carbonate. The mixture was shaken and allowed to stand for 100 min in darkness, following centrifugation at 15,000 $\times g$ for 3 min. The absorbance was measured at 760 nm with a microplate reader (Fluostar Omega, BMG Ortenberg, Germany). Results were expressed as mg gallic acid equivalents (GAE)/g of sample.

2.6. Antioxidant activity of seaweed and seaweed extracts

Antioxidant activity was measured in extracts using different assays: DPPH radical scavenging activity (DPPH), Oxygen Radical Absorbance Capacity (ORAC), FRAP (Ferric Reducing Ability of Plasma) and Trolox Equivalent Antioxidant Capacity (TEAC). In addition, total antioxidant activity was measured in samples without any extraction procedure (direct method) by the DPPH and TEAC methods.

DPPH was evaluated according to the procedure described by Brand-Williams et al. (1995) with modifications. A total of 0.1 mL of extract was added to 3.9 mL of 63.41 μ M DPPH methanolic solution. Absorbance was measured at 515 nm using a microplate reader (Fluostar Omega) for 60 min. Eight different concentrations (0.9–0.12 mM) of Trolox were used to make the calibration curve. Results were expressed as inhibition percentage (%).

FRAP was determined according to a procedure described earlier (Pereira et al., 2008). Results were expressed as μ mol Fe equivalent/g of sample.

ORAC was performed as described previously (Ou, Hampsch-Woodill, & Prior, 2001). A volume of 150 μ L fluorescein was placed in a 96-well black polystyrene plate, and 25 μ L of Trolox standard, sample or phosphate buffer as blank were added, all in duplicates. Samples, standards and blanks were incubated with fluorescein at 37 °C for 3 min before AAPH solution was added to initiate the oxidation reaction. Fluorescence was monitored over

35 min with a microplate reader (Fluostar Omega), using 485 nm excitation and 528 nm emission filters. Results were calculated using the areas under the fluorescein decay curves, between the blank and the sample, and were expressed as mmol Trolox Equivalents (TE)/g of sample.

TEAC analysis was used to evaluate the antioxidant capacity of the samples in a direct way, without extraction, as described earlier (Serpen, Capuano, Fogliano, & Gökmen, 2007). Ten milligrams of solid sample was mixed with 160 mL of ethanol:water (50:50, v:v). After, 1.6 mL ABTS solution was added to the sample. The mixture was incubated at 350 rpm during 30 min (Thermomixer Compact, Eppendorf, AG, Hamburg, Germany). Then, the sample was centrifuged at 15,330 x g during 2 min. The absorbance was measured at 730 nm with a microplate reader (Fluostar Omega,). Results were expressed as mg TE/g of sample.

2.7. Anti-hypertensive activity

ACE-inhibitory activity was performed as reported earlier (Shalaby, Zakora, & Otte, 2006). N-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycylglycine (FA-PGG) was dissolved at a concentration of 1.75 mmol/L in 50 mmol/L Tris-HCl buffer (pH 7.5) containing 0.3 mol/L NaCl. ACE solution of 0.25 units/mL was freshly prepared by adding 1 mL of purified water to a vial containing 0.25 units of enzyme. The assay was performed in a 96-well, clear, flat-bottomed polystyrene plate (Fluostar Omega). First, 10 µL of ACE solution and 10 µL of sample were placed in each well at room temperature. With an eight-channel pipette, 150 µL of preheated (37 °C, 15 min) substrate solution (FA-PGG) was added to each well to start the reaction. The microtiter plate was immediately transferred to a PowerWave microplate reader (BioTek, Winooski, VT, USA). The enzyme activity at 37 °C was based on the initial linear rate of change in absorbance at 340 nm, recorded every 3 min for 30 min. Captopril was used as a positive inhibitor for ACE at a concentration of 0.32 µmol/L. The ACE activity was

167 calculated as the slope of the decrease in absorbance at 340 nm (pA) and results expressed as
168 IC₅₀ (mg/mL).

169 2.8. *Anti-inflammatory activity*

170 Murine macrophage cell line RAW 264.7 (American Type Culture Collection,
171 Rockville, MD, USA) was grown in high-glucose DMEM supplemented with 10% FBS and
172 1% penicillin/streptomycin (5,000 U/mL) and maintained at a subconfluent density at 37 °C
173 under 5% CO₂/95% air atmosphere in a humidified incubator. All experiments were carried
174 out between passage 4 and passage 15 to ensure cell uniformity and reproducibility. For
175 inflammatory experiments, cells were seeded at a density of 5×10⁴ cells/well in 96-well plates
176 and allowed to grow overnight. The cells were pretreated with algae extracts (1-500 µg/mL)
177 dissolved in serum-free medium for 24 h. Then, the medium was removed and cells were
178 washed with PBS and elicited with 10 µg/mL LPS dissolved in serum-free medium for an
179 additional 24 h period. Finally, the media were collected and nitric oxide (NO) production,
180 tumor necrosis factor α (TNF-α) and prostaglandin (PG) D₂ detection were carried out.

181 The cytotoxic effect of algae extracts on RAW 264.7 cells was studied. Cell viability
182 was determined by using the MTT reduction assay. After cell treatments, culture medium was
183 removed, cells were washed with PBS and 200 µL of serum-free medium were added to each
184 well. Thereafter, 20 µL of a MTT solution in PBS (5 mg/mL) was added to each well and the
185 plates were incubated at 37 °C, 5% CO₂/95% air atmosphere for 2 h. Culture media was
186 aspirated and intracellular formazan crystals were solubilized using 200 µL of DMSO.
187 Absorbance was measured at 570 nm employing a microplate reader PowerWave™ XS
188 (BioTek) and compared against non-treated control cells (100% viable). The concentration of
189 algae extracts that showed no changes in cells viability compared with control were selected
190 for further studies.

Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reaction according to a previously described method (González-Montoya et al., 2018). Briefly, 100 μ L of DMEM were plated in 96-well plate and an equal amount of Griess reagent constituted by 1% (w/v) sulfanilamide and 0.1% (w/v) N-1-(naphthyl) ethylenediamine-di HCl in 2.5% (v/v) H_3PO_4 , was added. The plate was incubated for 5 min and the absorbance measured at 550 nm in a microplate reader (BioTek). The amount of NO was calculated using a sodium nitrite standard curve (0.25-10 μ g/mL).

For TNF- α analysis, a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Diacclone SAS, Besancon Cedex, France) was used. Briefly, 96-well plates were coated with TNF- α according to the manufacturer's instructions. Diluted biotinylated anti-TNF- α samples were added and incubated at room temperature for 3 h. After washing twice, streptavidin-HRP was added and incubated for 30 min. 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature for 12-15 min and the reaction was stopped by adding stop solution. The plate was read at 450 nm in a Synergy HT microplate reader (BioTek). TNF- α in culture medium was determined using the linear range of a standard curve (31.25-1,000 pg/mL) and expressed in pg/mL.

PGD₂ levels were determined using a prostaglandin D₂ ELISA (Cayman Chemical, Ann Arbor, Michigan, USA) according to the manufacturer's instructions. Plate was read at a wavelength of 405 nm using a Synergy HT microplate reader (BioTek). PGD₂ concentrations in samples from treated cell cultures were determined using the linear range of a standard curve. PGD₂ in culture medium was determined using the linear range of a standard curve (19.5-2,500 pg/mL) and expressed in pg/mL.

2.9. Triacylglycerol-lowering activity

In order to study the cytotoxic effects of the seaweed extracts in mature adipocytes, 3T3-L1 pre-adipocytes (American Type Culture Collection) were cultured in DMEM containing 10% Fetal Bovine Serum (FBS) (HyClone laboratories, South Logan, UT, USA). Two days after the adipocytes reached confluence (day 0), the cells were stimulated to differentiate for 2 days with DMEM containing 10% FCS, 10 µg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 µM dexamethasone. From day 4, differentiation medium was replaced by 10% FBS/DMEM medium containing 0.2 µg/mL insulin, and changed every two days until the cells were harvested. All media contained 1% penicillin/streptomycin (10,000 U/mL), and the media for differentiation and maturation contained 1% (v/v) of biotin and pantothenic acid. Cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere.

Once the cells reached the day 8 after differentiation, the mature adipocytes were grown in 6-well plates and incubated with insulin-free medium containing either 0.1% ethanol (control group) or seaweed extracts at 0.05, 0.1 and 0.5 mg/mL for 24 h. Then, cell viability was determined by the neutral red assay TOX-4 kit (Sigma-Aldrich).

For the measurement of triacylglycerol content, cells treated with seaweed extracts at 0.1 mg/mL for 24 h, were washed thoroughly with PBS and incubated with hexane/isopropanol (2:1, v/v) 3 times. Then, total volume was evaporated by vacuumed centrifugation and the pellet resuspended in 200 µL Triton X-100 in 1% distilled water. Finally, by means of sonication, triacylglycerols were disrupted and the content measured using a commercial kit (Infinity Triglycerides reagent, Thermo Electron Corporation, Rockford, IL, USA). In order to determine the protein amounts of the cells, these were lysed in 0.3 N NaOH, 0.1% SDS, and protein measured using the BCA reagent (Thermo Scientific, Rockford, IL, USA). Triacylglycerol content were expressed as mg/mg of protein and converted into arbitrary units.

2.10. Statistical analysis

All assays were performed at least in triplicate. Data represent the mean and standard deviation of three independent experiments (n = 6 for experiments in cell cultures and for the rest of experiments n = 3). Data were subjected to one-way ANOVA by using Statgraphics version 16.1.17 software (Statistical Graphics Corp., Rockville, USA). Differences between samples were compared by using a Duncan's multiple-range test at $p < 0.05$ probability level.

3. Results and Discussion

3.1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) characterization of the extracts

LC-MS profiles of all the seven algae extracts showed similar chromatographic elution of compounds with several compounds common among most of the samples (Figure 1). Several compounds including monomeric sugars along with lipophilic constituents were identified based on the predicted elemental composition following accurate mass measurements of the deprotonated, i.e. $[M-H]^-$, ions (Table 1). The $[M-H]^-$ ions for monomeric sugars at m/z 181.0682 ($C_6H_{13}O_6^-$) for mannitol, m/z 165.0796 ($C_6H_{13}O_5^-$) for fucitol, m/z 193 for glucuronic acid were observed in most of the samples. Ions at m/z 325 corresponding to deprotonated molecular ions of a disaccharide fucose-hexose were detected in *C. crispus*, *H. elongata* and *U. pinnatifida* (Table 1). As marine environment is a good medium for the synthesis of sulphated secondary metabolites in algae and the presence of sulphated oligosaccharides has been extensively described (Gonçalves, Ducatti, Duarte, & Nosedá, 2002; Gonçalves et al., 2005), a set of sulphated sugars with m/z 231 (xylitol sulphate), m/z 214 (dehydrated xylitol sulphate), m/z 261 (mannitol sulphate), m/z 265 (sodiated fucose sulphate) and m/z 259 (hexose sulphate) were also detected.

In addition to free-sugars, several fatty acids, lipids and prostaglandins (PG) were observed in the non-polar region of the LC-MS chromatogram. The tentative identities of

these non-polar molecules were reached through accurate measurements and tandem mass spectrometry (MS/MS) fragmentation patterns aided by existing literature (Sajiki & Kakimi, 1998; Santos et al., 2015). Deprotonated ions for fatty acids were observed at m/z 187 (nonanedioic acid), m/z 215.1681 ($C_{12}H_{23}O_3^-$), (hydroxydodecanoic acid), m/z 327 (oxo-dihydroxyoctadecenoic acid), m/z 297.2412 ($C_{18}H_{33}O_3^-$) (epoxyoctadecanoic acid) and m/z 329 (trihydroxyoctadecenoic acid). Ions at m/z 325 (dinor-11-PGF_{2α}), m/z 351.2184 ($C_{20}H_{31}O_5^-$) (PG_{E2/H2}), m/z 353 (PG_{F2α}), 367.2094 ($C_{20}H_{31}O_6^-$) (hydroxy PG_{F3α}), 369.2239 ($C_{20}H_{33}O_6^-$) (hydroxy PG_{F2α}) corresponding to a series of prostaglandins were detected in the extracts and have been reported previously in red algae (Sajiki & Kakimi, 1998). Ions at m/z 653.3768 ($C_{31}H_{57}O_{14}^-$) were tentatively identified as digalactosyl glyceryl palmitate and were present in all the samples except *H. elongata* and *U. pinnatifida* (Table 1). Phosphatidic acid (34:1) corresponding to ions at m/z 673, identified through accurate mass measurements and fragmentation pattern as reported by Kind et al. (Kind, Liu, Lee, Defelice, Meissen, & Fiehn, 2013), was detected only in *H. elongata* while Lysophosphatidylcholine (C16:0 – CH₃), found in macroalgae (Yao et al., 2015), with m/z 480.3080 ($C_{23}H_{47}O_7NP^-$) ions was present in *L. ochroleuca* and *P. palmata*.

Several antioxidants that have not been detected by the LC-MS/MS analysis performed in this study are likely to be present in the obtained extracts. In fact, Kellogg et al. (2015) explored the active subfractions purified from methanolic extracts obtained from six different seaweed species collected in Alaska to determine the phytochemical constituents. In that study, the authors confirmed the presence of fucophloroethol and phlorotannin oligomers. Further, other compounds such as monoglycosyldiacylglycerol (MGDG), featuring two eicosatetraenoic acid residues were also detected in the extracts (Kellogg et al., 2015).

3.2 Total phenolic content of seaweed extracts

Total phenols (TP) were determined in the methanolic extracts since polyphenols are common seaweed compounds (Duan, Zhang, Li, & Wang, 2006; Kuda et al., 2007; Lim, Cheung, Ooi, & Ang, 2002) and are associated with various biological properties. TP values ranged from 0.54 to 24.04 GAE/g among all the seaweed extracts (Figure 2A). *H. elongata* showed significant ($p \leq 0.05$) higher TP values compared to the other six seaweed species studied. The lowest TP values were observed in *U. pinnatifida* followed by *Ulva spp*, *L. ochroleuca*, *C. crispus*, *P. palmata* and *Phorphyra spp*. The values were lower than those reported by other authors (Rajauria, Jaiswal, Abu-Gannam, & Gupta, 2013), probably due to the different type of extraction and concentration of methanol used during the extraction procedure.

3.3. Antioxidant properties of seaweeds and seaweed extracts

DPPH. Seaweed extracts were evaluated in order to measure their DPPH-radical scavenging capacity. The highest DPPH reduction (87%) was obtained with *H. elongata* extract (Figure 2B). A significant positive correlation was observed between TP and DPPH ($r=0.9523$; $p<0.001$), suggesting that polyphenols may be the main components involved in the antiradical properties of the extract (Rajauria et al., 2013). Nevertheless, in accordance with the results reported by other authors, it is important to mention that the polysaccharides and covalently linked phenols can also be involved in this activity (Wang et al., 2016).

TEAC. Methanolic extracts displayed antioxidant activity as they were able to scavenge the ABTS^{•+} radical cation, showing significant differences among seaweed species (Figure 2C). The extract from *H. elongata* showed again the highest activity (107.215 $\mu\text{mol TE/g}$), meanwhile the rest of the extracts resulted in lower scavenging activities (3.48 to 15.85 $\mu\text{mol TE/g}$).

ORAC. This method, which determines peroxy radical scavenging activity, showed the same trend observed with other antioxidant methods; *H. elongata* had significantly ($p \leq 0.05$) highest antioxidant activity ($61.85 \mu\text{mol TE/g}$) than the rest of seaweeds evaluated (3.77 to $12.34 \mu\text{mol TE/g}$) (Figure 2D).

Results showed a significant ($p \leq 0.05$) correlation between phenol content and antioxidant activity (data not shown) although probably other compounds such as sulphated polysaccharides (fucans) and minor amounts of other sugars (including galactose, xylose, glucose, among others) in their composition may also be involved in these properties (Lim, Cheung, Ooi, & Ang, 2002) (Table 1).

FRAP. Reducing power of the extracts was analyzed, since it is frequently related to the degree of hydroxylation and extent of conjugation in polyphenols. *H. elongata* showed higher activity ($1.44 \pm 0.88 \mu\text{mol Fe/g}$) than the other seaweed extracts, where values ranged from 0.06 to $0.14 \mu\text{mol Fe/g}$. (Figure 2E). The lowest values appeared in *U. pinnatifida* ($0.06 \mu\text{mol Fe/g}$) and the other seaweed extracts showed FRAP values close to *H. elongata*.

The antioxidant activity measured by all those methods could be attributed mainly to the presence of phenolic compounds in seaweeds extracts (Vo Dinh, Saravana, Woo, & Chun, 2018). However, some sulfated sugars identified in the seaweeds extracts obtained in the present work (xylitol sulphate, mannitol sulphate, anhydrous xylitol sulphate, hexose sulphate and sodiated fucose sulphate) that may be also contributing to the antioxidant activity of these extracts. This hypothesis is supported by different studies that have found antioxidant activity of seaweed sulfated polysaccharides by different *in vitro* methods such as ABTS radical scavenging (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2014), DPPH radical scavenging (Seedevi, Moovendhan, Viramani, & Shanmugam, 2017), FRAP (Xie, Wang, Shen, Nie, Gong, Li, et al., 2016), lipid peroxide inhibition (Ben Gara, Ben Abdallah Kolsi, Chaaben,

Hammami, Kammoun, Paolo Patti, et al., 2017), and superoxide and hydroxyl radical scavenging assays (Abu, Jiang, Ueno, Okimura, Yamaguchi, & Oda, 2013; Deng, Xu, Fu, Chen, & Xu, 2015), among others.

Antioxidant capacity was also measured through direct methods (DPPH and TEAC) (Figure 3A and 3B). Results showed, as it occurred in extract-based methods, higher antioxidant activity of *H. elongata* as compared to the other seaweeds studied. However, DPPH reduction assay revealed relatively high antiradical effect of *U. pinnatifida*, *L. ochroleuca* and *P. palmata*, higher than 50% reduction, which could explain that other non-soluble compounds may also contribute on their total antioxidant activity.

3.3. Antihypertensive properties of seaweed

Inhibition of ACE-I is a well-established approach in the treatment of hypertension, and because of that, many authors have screened the potential of seaweeds in inhibiting this enzyme. Cha et al. (2006) screened the *in vitro* ACE-I inhibitory activity of methanol and aqueous extracts from twenty-six red Korean algae, obtained at 20 °C or at 70 °C. The authors found several potential extracts, with IC₅₀ values for ACE-I in the range of 12.21–124.69 µg/mL. In our study, among the tested seaweeds, only *H. elongata* showed ACE inhibitory potential (IC₅₀=65 µg/mL), with an active concentration in the range previously reported by Cha et al (2006). Furthermore, the range of the IC₅₀ observed for *H. elongata* is only ten-fold higher (lower activity) than those found in works applying proteolytic enzymatic digestions for screening ACE-I-inhibitory bioactive peptides (Cardoso et al., 2015; Suetsuna, Maekawa, & Chen, 2004).

3.4. Anti-inflammatory activity of seaweed extracts

In diet-induced obesity, adipose tissue is in a chronic low-grade state of inflammation predisposing the development of MetS. This event is driven by the infiltration of macrophages

into adipose tissue, which, together with adipocytes, perpetuate a cycle of macrophage recruitment and secretion of free fatty acids and deleterious cytokines/chemokines that predispose to development of MetS (Dandona et al., 2005). Newly released inflammatory cytokines drives the inflammatory responses to upregulate pro-inflammatory genes expression such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) and contribute to the progression of systemic inflammation (Dandona et al., 2005). The former is involved in NO synthesis and the latter represent a key enzyme in the synthesis of lipid inflammatory mediators that include prostaglandins. One potential strategy to reduce obesity-induced inflammation is to inhibit the activity and expression of these proinflammatory enzymes (Gu et al., 2014). Therefore, we evaluated the anti-inflammatory potential of seaweed extracts in a model of LPS-induced RAW264.7 macrophages in culture. Methanolic extracts from the seven seaweed species were evaluated for their cytotoxic effect on RAW 264.7 macrophages. Results showed that the seaweed extracts did not significantly ($p < 0.05$) affect cell viability ($\geq 90\%$ of the control) indicating that the tested concentration did not damage cell integrity (Figure 4A). This non-toxic extract dose was assayed for their inhibitory potential against three common pro-inflammatory markers NO, TNF- α and PGD₂. Previous studies have demonstrated that PGD₂ is the most abundant cyclooxygenase 2 (COX-2) product in LPS-stimulated RAW264.7 macrophages (Buczynski et al., 2007). NO and PGD₂ were determined in LPS-stimulated RAW264.7 macrophages as a biomarker of iNOS and COX-2 activity.

To investigate if seaweed extracts exert anti-inflammatory properties, RAW 264.7 cells were pre-treated with 500 $\mu\text{g/mL}$ algae extracts for 24 h and subsequently stimulated with 10 $\mu\text{g/mL}$ LPS for 24 h. Production of NO, PGD₂, and TNF- α was measured in the culture medium. When LPS was administered to non-pretreated macrophages the NO production, and PGD₂ and TNF- α secretion was increased significantly ($p < 0.05$) from the basal level after 24 h incubation (Figures 4B-4D), indicating the successful activation of cells by LPS stimulation.

The results showed that most of the extracts evaluated were able to reduce significantly ($p<0.05$) the NO production in LPS-activated macrophages (>50% of inhibition) (Figure 4B). This reduction was notably higher in cells treated with *U. pinnatifida* and *H. elongata* extracts (~92.5% and ~74.8% of inhibition, respectively). On the other hand, the *Porphyra* spp., *U. pinnatifida*, *C. crispus*, and *L. ochroleuca* extracts, at a dose of 500 µg/mL, exhibited a significant reduction ($p<0.05$) in the level of LPS-induced PGD₂ and TNF-α (Figure 4C and 4D). Noteworthy, cell treatment in presence of *U. pinnatifida* extract was extremely effective in the inhibition of pro-inflammatory PGD₂ and TNF-α (~96.5% and ~77.5%, respectively). However, even though *H. elongata* and *P. palmata* extracts were significantly ($p<0.05$) effective reducing the LPS-induced PGD₂ secretion, they did not evoke a significant ($p>0.05$) reduction on the TNF-α levels (Figure 4D). Differences in the anti-inflammatory activity of seaweed extracts could be due to variations in composition of bioactive compounds.

Our results are consistent with previous studies showing the anti-inflammatory effects of seaweed extracts from brown, green and red seaweed species (Joung et al., 2017; Kellogg et al., 2015). All species (*Sargassum serratifolium*, *Dictiopteris divaricate*, *Dictiopteris prolifera*, *Prionitis cornea*, *Grateloupia lanceolate*, *Grateloupia filicina*, *Fucus distichus*, *Alaria marginate*, *Saccharina groenlandica*, and *Saccharina latissimi*, *Ecklonia stolonifera*) strongly inhibited NO production, decreased secretion of PGE₂ and, TNF-α and interleukin (IL)-6 and protein expression of inducible NO synthase and COX-2. Fatty acids and lipid derivatives from different marine algae species have shown anti-inflammatory potential in LPS-activated RAW 264.7 macrophages (Dang et al., 2008; Lee et al., 2009). The anti-inflammatory potential of *H. elongata* and *U. pinnatifida* could be supported by the presence of dihydroxy-oxo-octadecenoic, trihydroxyoctadecenoic and nonanodieic acids found in methanolic extracts. The structure-activity relationship of oxygenated fatty acids have revealed that the presence of α, β-unsaturated carbonyl moiety significantly enhances the

anti-inflammatory activity of LPS induced RAW cells along with some other minor structural features such as; chain length, terminal functional group and position of enone groups (Dang et al., 2008).

Other bioactive compounds not identified herein could have been contributed to the overall anti-inflammatory activity of seaweed extracts. For instance, seaweed phlorotannin oligomers ranging from 9 to 20 have been shown with the potential to inhibit inflammatory markers, including prostaglandin synthesis, through a potential mechanism of down regulation of the expression of Toll-like receptors such as TLR4 and TLR9 (Kellogg et al., 2015). In particular, phloroglucinol, eckol, dieckol, 7-phloroeckol, phlorofucofuroeckol A and dioxinodehydroeckol from brown seaweed species has been reported as pronounced inhibitors of pro-inflammatory enzymes and cytokines in LPS-stimulated macrophages (Jung et al., 2013). More recently, sargahydroquinoic acid, argachromenol, and sagaquinoic acid were identified as the main anti-inflammatory compounds in the brown algae *Sargassum serratifolium* (Joung et al., 2017). Sulfated polysaccharides such as fucoidans are also an interesting group of anti-inflammatory compounds abundant in brown seaweeds. There are at least two distinct forms of fucoidan: F-fucoidan, which is >95% composed of sulfated esters of fucose, and U-fucoidan, which is approximately 20% glucuronic acid. Current findings confirm that isolated fucoidans from brown seaweeds species inhibit NO production and expression of PGs through the subsequent downregulation of iNOS and COX-2 expression in LPS-stimulated RAW264.7 macrophages and in zebrafish larvae (Jeong et al., 2017). *H. elongata* and *U. pinnatifida* extracts were composed by some constituents of fucoidans including monomers of glucuronic acid, galactose-fucose and sodiated fucose sulphate, however, there is no evidence so far showing the anti-inflammatory effect of these small compounds.

3.6 Adipocyte viability and triacylglyceride-lowering activity

433 *P. palmata* seaweed extract showed cytotoxic effects when added to the mature
434 adipocytes in the three tested doses (0.05, 0.1 and 0.5 mg/mL) (Figure 5A). Regarding all the
435 other extracts, no cytotoxic effects were observed under the aforementioned extract doses.
436 Other authors did not find cytotoxic effects of extracts from seaweeds, other than those used
437 in this study, up to a 48 h incubation period in 3T3-L1 preadipocytes at a dose of 0.1 mg/mL
438 (Ko et al., 2013). Further, in a study performed in mature adipocytes incubated for 48 h with
439 fucoxanthin, a major marine carotenoid isolated from brown seaweeds such as *U. pinnatifida*,
440 at doses up to 20 µM, no cytotoxic effect was found (Kang et al., 2011). However, Kang et al.
441 (2016b), in their extensive study performed with a great number of brown, green and red
442 seaweeds obtained from Jeju Island (Korea), reported a cytotoxic effect for an ethanolic
443 extract obtained from some seaweeds, including *U. pinnatifida*, after incubating preadipocytes
444 for 48 h with 0.1 mg/mL of the extract.

445 Taking into account the cell viability assay results, the 0.1 mg/mL extract dose was
446 selected to analyze the effects of the studied seaweeds regarding their triacylglyceride
447 lowering activity. The extracts obtained from *P. palmata*, *Ulva spp.*, *U. pinnatifida* and *H.*
448 *elongata* induced significant reductions (42.96%, 49.96%, 36.35% and 52.0% respectively) in
449 triacylglycerol content in mature 3T3-L1 adipocytes after 24 h incubation (Figure 5B).

450 Obesity and overweight processes are determined not only by adipocyte hyperplasia
451 (increasing the number of adipocytes) but also by adipose tissues grow by hypertrophy
452 (increasing the size of their adipocytes). It seems that a “critical” adipocyte size exists,
453 triggering the recruitment of preadipocytes that eventually differentiated into mature
454 adipocytes (Faust, Johnson, Stern, & Hirsch, 1978). In this line, some extracts obtained from
455 different types of seaweeds, including one from *U. pinnatifida*, have also shown triglyceride
456 reducing effect in maturing adipocytes (Kang et al., 2016b). These authors, in a study
457 performed in 3T3-L1 preadipocytes with an extract from *Gelidium amansii* devoted to explore

the mechanisms underlying the inhibition of intracellular lipid accumulation, demonstrated that an ethanolic extract at a concentration of 100 µg/mL decreased protein expression levels of some adipogenic-specific proteins (PPAR γ , C/EBP α , and SREBP-1), compared to the non-treated cells (Kang et al., 2016a). Furthermore, fucoxanthin, marine carotenoid found in edible brown seaweeds such as *U. pinnatifida* and *Laminaria japonica*, reduced the expression of PPAR γ , C/EBP α , and SREBP1c during the intermediate and late stages of adipocyte differentiation (Kang, et al., 2011). Similar effects were found by Yim et al. (2011) in a study devoted to investigate the effects of fucoxanthin and its metabolites, fucoxanthinol and amarouciaxanthin A, on 3T3-L1 preadipocyte differentiation. These authors concluded that amarouciaxanthin A reduces 3T3-L1 adipocyte differentiation through the down-regulation of PPAR γ and C/EBP α mRNA expression (Yim, et al., 2011). Some of these compounds are likely to be present in our extracts since Kellogg et al (2015) also using an aqueous methanolic solution detected fucophloroethol and phlorotannin oligomers in a sub-fraction of the obtained extracts, and show them as responsible for the reduction in triacylglyceride accumulation observed in adipocytes (Kellogg et al., 2015).

To the best of our knowledge, this is the first study conducted with mature adipocytes aimed to analyze the viability and triacylglyceride accumulation after seaweed extract treatment. Considering that systemic metabolic dysfunction - unhealthy obesity- is primarily associated with mature adipocyte hypertrophy (Lessard et al., 2014), extracts from *Ulva spp.*, *P. palmata*, *U. pinnatifida* and *H. elongata* could be considered as potential tool in reducing obesity.

4. Conclusions

This is the first study that evaluated the multifunctional potential of mostly consumed seaweeds with the objective to identify a species displaying an array of relevant bioactivities

to attenuate the cardiometabolic risk factors of MetS. Among seaweeds, *H. elongata* showed the better potential to be used as raw material in the development of new functional foods for management of MetS. Methanolic extracts of this seaweed species showed the highest phenolic content, antioxidant activity and angiotensin converting enzyme I inhibitory activity *in vitro*. In addition, *H. elongata* effectively reduced LPS-induced inflammation in RAW 264.7 macrophages and triglyceride accumulation in 3T3-L1 mature adipocytes. Phytochemical investigations by LC-MS indicated the presence of monomeric sugars/sulfated sugars and lipids in the bioactive extracts.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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703 **Figure legends**

704 **Figure 1.** HPLC-MS/MS chromatograms for *Porphyra purpurea* (A), *Undaria pinnatifida* (B), *Ulva* spp. (C),
705 *Himanthalia elongata* (D), *Chondrus crispus* (E), *Laminaria ochroleuca* (F) and *Palmaria palmata*
706 (G).

707
708 **Figure 2.** Total phenolic compounds and antioxidant activities of *Porphyra* spp., *Undaria pinnatifida*, *Ulva*
709 spp., *Himanthalia elongata*, *Chondrus crispus*, *Laminaria ochroleuca* and *Palmaria palmata* were
710 assessed using the following methods: TP (A), DPPH (B), TEAC (C), ORAC (D) and FRAP (E).
711 Data were expressed in $\mu\text{mol GAE (GAE)}/\text{g}$ for TP, % of reduction for DPPH, $\mu\text{mol TE}/\text{g}$ for
712 TEAC, $\mu\text{mol TE}/\text{g}$ for ORAC and $\mu\text{mol Fe}/\text{g}$ for FRAP. Data are the mean $\pm$ standard deviation of
713 three replicates analyzed in duplicate. Different letters indicate statistical differences ($P < 0.05$,
714 Duncan's test).

715
716 **Figure 3.** Direct measurement of total antioxidant capacity (QUENCHER) using DPPH and TEAC methods
717 for *Porphyra* spp., *Undaria pinnatifida*, *Ulva* spp., *Himanthalia elongata*, *Chondrus crispus*,
718 *Laminaria ochroleuca* and *Palmaria palmata*. Data were expressed as % of reduction for DPPH and
719 $\mu\text{mol TE}/\text{g}$ for TEAC. Data are the mean $\pm$ standard deviation of three replicates analyzed in
720 duplicate. Different letters indicate statistical differences ($P < 0.05$, Duncan's test).

721
722 **Figure 4.** Cytotoxic effect of seaweed extracts in RAW264.7 macrophages (500 $\mu\text{g}/\text{mL}$) (A). The results are
723 expressed as percentage of control and are represented by mean $\pm$ SD ($n = 6$). Effect of algae
724 extracts (500 $\mu\text{g}/\text{mL}$) on NO production (B), PGD_2 (C), and $\text{TNF-}\alpha$ secretion (D) in LPS-stimulated
725 RAW 264.7 macrophages. The results are expressed as percentage of control and are represented by
726 mean $\pm$ SD ($n = 6$). Significant decreased ($p < 0.05$) NO, PGD_2 , and $\text{TNF-}\alpha$ production is depicted
727 with asterisk as compared to cells treated with LPS (10 $\mu\text{g}/\text{mL}$).

728
729 **Figure 5.** Cytotoxicity and triacylglyceride-lowering activity of seaweed extracts. 3T3-L1 mature adipocytes
730 were treated for 24 h with 0.05, 0.1 or 0.5 mg/mL of different seaweed extracts, and their viability
731 was evaluated by the neutral red assay (A). Triacylglycerol amounts in 3T3-L1 mature adipocytes
732 incubated for 24h with different seaweed extracts at a dose of 0.1 mg/mL (B). Data represent the
733 mean $\pm$ SD ($n = 6$). Asterisk indicate statistically significant differences ($P < 0.05$) compared with
734 control condition.

Table 1

Table 1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Identification of compounds based on *m/z* and fragmentation pattern for *Himanthalia elongata* and *Undaria pinnatifida*

ALGAE	<i>t_R</i>	[M-H] ⁻ <i>m/z</i>	Mass Error (mDa)	Molecular Formula	MS/MS fragment ions (<i>m/z</i>)	Tentative ID
<i>Himanthalia elongata</i>	1.4	325.1113	-2.2	C ₁₂ H ₂₁ O ₁₀	178, 146	Fucose-Galactose
	2.7	231.0139	-3.6	C ₅ H ₁₁ O ₈ S	151	Xylitol sulphate
	6.9	193.0339	-0.9	C ₆ H ₉ O ₇	149, 121	Glucuronic acid
	7.1	261.0241	-3.9	C ₆ H ₁₃ O ₉ S	165, 129	Mannitol sulphate
	8.2	187.0952	-1.8	C ₉ H ₁₅ O ₄	-	Nonanedioic acid
	10.2	327.2205	3.4	C ₁₈ H ₃₁ O ₅	309, 291, 229, 211, 171	Dihydroxy-oxo-octadecenoic acid
	10.6	329.2343	1.5	C ₁₈ H ₃₃ O ₅	311, 293, 211, 171, 139, 127	Trihydroxyoctadecenoic acid
	11.2	353.2354	2.6	C ₂₀ H ₃₃ O ₅	317, 273, 235, 127	Prostaglandin F _{2α}
	13.2	673.4819	1.1	C ₃₇ H ₇₀ O ₈ P	593, 297, 153, 211, 137, 97, 80	Phosphatidic acid (34:1)
<i>Undaria pinnatifida</i>	6.2	214.0154	0.7	C ₅ H ₁₀ O ₇ S	150, 134	Anhydrous xylitol sulphate
	6.9	193.0339	-0.9	C ₆ H ₉ O ₇	149, 121, 105	Glucuronic acid
	7.9	259.0149	2.5	C ₆ H ₁₁ O ₉ S	199, 153	Hexose sulphate
	8.2	187.0952	-1.8	C ₉ H ₁₅ O ₄	169, 125, 123	Nonanedioic acid
	8.7	259.0149	2.5	C ₆ H ₁₁ O ₉ S	199	Hexose sulphate
	10.2	264.9995	0.1	C ₆ H ₁₀ O ₈ SNa	190	Sodiated fucose sulphate
	10.7	325.1988	-2.7	C ₁₈ H ₂₉ O ₅	307, 199, 183, 171, 129	Dinor PGF _{2α}

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.