SEAWEED FOR HEALTH CONFERENCE, GALWAY

Fatty acid composition, TLC screening, ATR-FTIR analysis, anti-cholinesterase activity, and in vitro cytotoxicity to A549 tumor cell line of extracts of 3 macroalgae collected in Madeira

N. Nunes[1,2] (ID) · G. P. Rosa[3] · S. Ferraz[1] · Maria Carmo Barreto[3] · M. A. A. Pinheiro de Carvalho[1]

Abstract

Three macroalgae collected at Madeira Island were included in this study to determine their potential for drug, nutraceutical, food, or supplement application. Fatty acid content was higher in *Zonaria tournefortii* (12.32 mg g^{-1} dw) with 16.58% of PUFAs, eicosapentaenoic acid (C20:5ω3), and arachidonic acid (20:4ω6) having concentrations of 2.59 and 1.17%, respectively. The anti-thrombogenic and anti-atherogenicity potential was higher for *Z. tournefortii* due to relevant fatty acids in the biochemical composition this macroalgae. Lipid classes were assessed in the lipid extract and neutral lipids (NL) were in higher yield in *Asparagopsis taxiformis* (51.16%) and lower in *Z. tournefortii* (26.96%). The glycolipids (GL) were between 36.03 and 16.11% in *Z. tournefortii* and *Ulva lactuca*. Phospholipids (PL) fraction varied from 35.91 and 31.60% in *A. taxiformis* and *Z. tournefortii*. TLC screening identified that *U. lactuca* contains phytol and cholesterol in its NL, digalactosyldiacylglycerol in its GL, and cardiolipin and L-α-phosphatidylcholine in its PL. *Zonaria tournefortii* contains phytol and cholesterol in its NL classes, and the PL classes contain L-α-phosphatidylethanolamine and 1-(3-*sn*-phosphatidyl)-*rac*-glycerol. The macroalgae *A. taxiformis* revealed cholesterol in its NL fraction and the same phospholipids as *Z. tournefortii* in its PL fraction. ATR-FTIR analysis enabled a "fingerprint" spectra and important sulfation absorption bands were identified, revealing the functional polysaccharides within these macroalgae. Anti-cholinesterasic activity was assessed in *A. taxiformis*, with a low IC$_{50}$ for AChE (8.92 ± 0.43 μg mL^{-1}) and BuChE (13.96 ± 0.32 μg mL^{-1}), demonstrating dual inhibitory activity, justifying the interest to identify the active principle which may be the scaffold of a novel drug.

Keywords Seaweeds · *Asparagopsis taxiformis* · *Ulva lactuca* · *Zonaria tournefortii* · Lipids · PUFAs · MUFAs · SFAs

Introduction

Nowadays, intensive research is conducted worldwide to explore and determine the nutritive, nutraceutical, pharmaceutical, and cosmeceutical potential of innumerous extracts from organic materials. Seaweeds have been included in this effort

✉ N. Nunes
nuno.nunes@staff.uma.pt

1   ISOPlexis, University of Madeira Campus da Penteada, 9050-290 Funchal, Madeira, Portugal

2   UBQ II, Unidade de Bioquímica, Lda. Rua Visconde de Anadia, Edifício Anadia 5° Andar CC, 9050-020 Funchal, Madeira, Portugal

3   cE3c–Centre for Ecology, Evolution and Environmental Changes/ Azorean Biodiversity Group and Faculty of Sciences and Technology, University of Azores, 9501-801 Ponta Delgada, Portugal

due to their intrinsic biochemical composition, derived from evolutionary adaptation to the harsh conditions of high salt concentration, desiccation and herbivory, and extreme variations in sunlight and temperature fluctuation. The sea currents and wave energy also cause motional pressure to these organisms, since they must remain attached to the substrate, subjected to additional environmental stress, adjusting the metabolic network and developing new metabolites to permit adaptation to this variable habitat (Mišurcová et al. 2011).

Seaweeds have a low lipid content but are considered a potential source of functional lipids due to their enormous stock in coastal waters. Among total lipids (TL), lipid composition and quantity varies according to species, geographical origin, and environmental conditions (Miyashita et al. 2013). TL are further divided in lipid classes such as glycolipids (GL), triacylglycerols (TAG), and phospholipids (PL). In these classes, fatty acids exist as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids

(PUFAs). These last (PUFAs) are essential to human nutrition, since algae produce PUFAs by de novo synthesis and which cannot be biosynthesized by almost all heterotrophic organisms, having to be ingested in the diet. Nowadays, seaweeds are also considered as functional food and nutraceuticals with health benefits, since they are able to reduce the risk of cardiovascular diseases (CVD), diabetes, osteoporosis, and cancer (Mišurcová et al. 2011). GL is the most common lipid group and includes monogalactosyl-diacylglycerols (MGDG), digalactosyl-diacylglycerol (DGDG), and sulfoquinovosyl-diacylglycerol (SQDG) (Holdt and Kraan 2011). GLs are important in photosynthetic membranes of higher plants, in algae and bacteria. In brown seaweeds, GLs are the primary membrane lipids, with high concentration of specific long chain fatty acids, which include stearidonic acid (18:4n-3) (SDA), eicosapentaenoic acid (20:5n-3) (EPA), and arachidonic acid (20:4n-6) (ARA) (Miyashita et al. 2013). SDA is formed by the desaturation of α-linolenic acid (ALA), and when consumed increases the level of eicosapentaenoic acid (EPA) in erythrocyte membranes (Lemke et al. 2013). Epidemiological experiments demonstrated that ingesting long chain ω3 fatty acids decreases cardiovascular diseases, mainly heart attacks (Albert et al. 1998). The ω6 fatty acids are particularly important in biological systems. ARA participates in the immune response, prevents thrombosis, helps brain function, and together with docosahexaenoic acid (DHA), is correlated with a reduction in age-related disorders of the brain and cognitive functions (Hoffman et al. 2009; Kiso 2011). These are the main components of cell membranes and participate in neuron structure in the central nervous system (Miyashita et al. 2013).

Macroalgae are also an interesting source of novel molecules which may be the scaffolds of more efficient drugs, namely against age-related diseases (Barbosa et al. 2014). Alzheimer's disease (AD) is a progressive neurodegenerative disorder, affecting roughly 2% of the population in industrialized countries, which is characterized by synapse degeneration and neuronal death in regions of the brain responsible for learning and memory processes (Mattson 2004). Two main strategies exist to improve the cholinergic function in AD, which are stimulating cholinergic receptors or increasing the availability of acetylcholine into the neuronal synaptic cleft, inhibiting the enzyme acetylcholinesterase (AChE, E.C. 3.1.1.7) (Howes et al. 2003). Several pharmaceuticals are available for this second strategy and include tacrine, rivastigmine, galantamine, and donepezil; however, the use of these drugs only slows the progress of the illness and causes negative side effects, highlighting the need for new sources of anti-acetylcholinesterasic compounds (Arruda et al. 2012). The inhibition of butyrylcholinesterase or pseudocholinesterase (BuChE, EC 3.1.1.8) may also contribute to the treatment of AD. BuChE is produced mainly in the liver and catalyzes butyrylcholine more rapidly, although it is also able to hydrolyze acetylcholine (Colovic et al. 2013).

Seaweed extracts and pure compounds have been researched for their cytotoxic effect in human A549 lung cancer cellular lines, due to their unique chemical structures that could be used as novel drugs (Alwarsamy et al. 2016). Lung cancer is the most prominent cause of cancer deaths, accounting more than one million deaths worldwide per annum (Lee et al. 2011). This cancer development is characterized by proliferation of anaplastic cells without restraint, invading and metastasizing to the surrounding tissues and organs (Marudhupandi et al. 2015). Several factors are responsible for this illness and include unhealthy diet, inherited genetic mutations, drug use, hormones, environmental toxins, infectious organisms, and immune conditions, the treatment being the obliteration of the tumor cells (Gutiérrez-Rodríguez et al. 2018). Standardized treatment of this malignancy is the application of synthetic compounds in chemotherapy, which attacks the cancer cells but at the same time is cytotoxic to normal cells (Alwarsamy et al. 2016). It is therefore of the utmost importance to discover new sources of bioactive compounds that can reduce this malignancy without the side effects.

This work intended to determine the potential of these 3 seaweeds, due to their significant abundance in Madeira Island. This is an effort to search new sources of bioactive compounds that would help develop purification strategies to research for new pharmaceutical compounds, namely displaying anti-cholinesterasic and/or cytotoxic activity against A549 tumor cell lines. This study will also determine if these macroalgae are good sources of essential fatty acids. These assessments could permit to develop new drugs, new nutraceuticals or to envision new food sources with multiple health applications.

Materials and methods

Collection of samples

The chlorophyte macroalga *Ulva lactuca* Linnaeus was collected on 15 of May 2017, in "Santa Cruz" at coordinates 32° 41′ 27″ N and 16° 46′ 25″ W. The rhodophyte *Asparagopsis taxiformis* (Delile) Trevisan was collected on 8 of September 2017 and the phaeophyte *Zonaria tournefortii* (J.V. Lamouroux) Montagne collected on 7 of July 2017 in "Reis Magos" beach, coordinates 32° 38′ 48″ N and 16° 49′ 26″ W in a 10 m maximum depth dive of the Madeiran archipelago. Samples were transported in seawater and gently rinsed with filtered fresh water. Afterwards, a primary drying was applied in which seaweed was frozen at − 35 °C and freeze-dried for 5 days under reduced pressure (4×10^{-4} mbar), with a cooling trap set at − 56 °C. Samples were milled to 200 mesh particle size, vacuum packed, and stored at − 35 °C until use.

Fatty acid determination

The fatty acid (FA) composition was determined according to Lepage and Roy (1986) with the modifications suggested by Cohen et al. (1988) and assessed as FA methyl esters (FAME). Initially, for the conversion of FAs to FAMEs, a mixture of ethyl acetate-methanol (1:19 v/v) was added to the dried samples. These were incubated at 80 °C for 1 h and followed by an extraction using heptane. The analytical apparatus consisted of a gas chromatograph (Agilent HP 6890), fitted with a mass selective detector (Agilent 5973) and a capillary column DB-225J&W (30 m × 0.25 mm inner diameter, 0.15-μm film thickness), also from Agilent. Chromatographic settings were: helium was used as the carrier gas set to a flow rate of 2.6 mL min^{-1}, initial temperature of the oven was set to 35 °C for a period of 0.5 min, subsequently incremented by 25 °C min^{-1} to 195 °C, followed by 3 °C min^{-1} to 205 °C and 8 °C min^{-1} to achieve a maximum temperature of 230 °C for a period of 3 min. Injector temperature was set to 250 °C and the transfer line set to 280 °C with the split ratio at 1:100. FAMEs were identified, comparing the retention times and mass spectra fragmentation to standards (bacterial acid methyl esters CP mix and Supelco 37 component FAME mix). The internal standard was heneicosanoic acid (C21:0). Two measures of each sample were performed, presented as mean values ± standard deviation (SD) of FAME and results expressed in mg g^{-1} of dry weight (DW).

The index of thrombogenicity (IT) and the index of atherogenicity (IA), in which lower results indicate a lower risk to develop those pathologies, was calculated as proposed by Ulbricht and Southgate (1991) using the following equations:

$$IT = (14:0 + 16:0 + 18:0)/$$
$$\left[(0.5 \times \Sigma MUFA + 0.5 \times PUFA\omega6 + 3 \times PUFA\omega3) + (PUFA\omega3/PUFA\omega6))\right]$$

$$IA = \left[(12:0 + (4 \times 14:0) + 16:0)\right]/$$
$$\left[\Sigma MUFA + PUFA\omega6 + PUFA\omega3\right]$$

Extraction and fractionation

Lipid extraction

The methodology of Folch et al. (1957), with some modifications, was used to perform the lipid extractions (LE) from the macroalgae. Initially, 2 g of freeze-dried milled macroalgae were mixed with 40 mL of $CHCl_3$:MeOH (2:1). Subsequently, it was sonicated for 10 min at 37 kHz at 100% and centrifuged at 7197xg at room temperature. The liquid extract was filtered with a 8-μm pore filter paper, added to 8 mL of 0.9% NaCl solution, vortexed, and centrifuged at

470xg for phase separation. The upper phase was removed and discarded, leaving the lipophilic phase. The majority of the solvent was evaporated in a rotary evaporator and the residual solvent evaporated in an oven at 35 °C overnight and weighed. These extracts will from now on be abbreviated as LE.

Ethanol extraction

The ethanol extracts (EE) were performed with 96% ethanol, using a Timatic semiautomatic extractor (Technolab, Spello, Italy), set at a constant pressure and static phase. The program was set as follows: solvent capacity 1 L, solvent to biomass ratio 50:1, compression time 3 min, decompression time 6 min, minimum pressure 6 bar, compression pressure 8.5 bar at room temperature (± 23 °C). The number of cycles was set to 12 cycles, which corresponded to 108 min for each extraction. Each sample of 20 g was placed in a 100-μm mesh bag and placed inside a 0.5-L extracting vessel. In the end of each extraction, the liquid extracts were filtered through a 8-μm Whatman filter and were partly evaporated in a rotary evaporator and dehydrated completely in an oven, at 35 °C until complete dryness. After, these were stored under vacuum at − 35 °C until use.

Lipid classes

LE were fractioned into neutral (N), glycolipid (GL), and phospholipid (PL) classes. Initially, 50 mg of extract were solubilized in 2 mL of chloroform and applied to a glass column with 10 g of 200 to 400 mesh silica particles. Silica was previously packed with chloroform, with quartz sand beneath and underneath for protection. Fractionation was performed using 100 mL of a specific solvent for each class. Neutral lipids and sterols were extracted with chloroform, glycolipids with acetone, and phospholipids with methanol. For each extraction, the corresponding solvent containing the lipid class was collected and dried, initially in a rotary evaporator, afterwards in an oven at 35 °C. The residue was weighted and stored at − 35 °C until use.

Thin layer chromatography

The separated lipid classes were assessed using thin layer chromatography (TLC) with Macherey-Nagel Pre-coated TLC sheets POLYGRAM SIL G/UV$_{254}$ (0.20 mm silica gel 60). The TLC mobile phase for neutral lipids was hexane:diethyl ether:acetic acid (85:15:0.1, v/v), and TLC plate was revealed with vanillin solubilized in sulfuric acid (1:100, w/v), with posterior heating at 120 °C in an oven until complete dryness of the TLC plate. The standards were cholesterol acetate, cholesterol, β-carotene, and phytol. The mobile phase for glycolipids was dichloromethane:methanol

(85:15, v/v) and the TLC plate was revealed with α-naphthol:sulfuric acid:water:ethanol (2.4:10:10:80, w/v), and the TLC plate heated at 120 °C for 3 to 5 min, until pink and purple spots were detected. The standard used was digalactosyldiacylglycerol (DGDG). Phospholipids were separated by polarity and charge. The mobile phase was chloroform:methanol:ammonium hydroxide (65:25:4, v/v), revealed with sprayed 10% copper and 8% phosphoric acid solubilized in water, and placed in an oven at 120 °C until spots appeared. The standards used were L-α-phosphatidylcholine, L-α-phosphatidylethanolamine, 1-(3-*sn*-phosphatidyl)-*rac*-glycerol sodium salt, 1,2-diacyl-*sn*-glycerol-phospho-L-serine, cardiolipin sodium salt, and lysophosphatidylcholine. The retention factor (Eq. 1) was calculated in millimeters for the separated compounds within each lipid class, as this is calculated by dividing the distance traveled by the compound and the distance traveled by the solvent front.

Bioactivities

Anti-cholinesterase activity

The assays to assess anti-acetylcholinesterase (AChE) and anti-butyrylcholinesterase activities (BuChE) were adapted from Ellman et al. (1961) modified by Arruda et al. (2012). In each microplate, serial dilutions of the extracts were prepared in 100 mM phosphate buffer pH 8, in order to obtain concentrations between 0 and 150 μg L^{-1}. Afterwards, 0.25 U mL^{-1} acetylcholinesterase or butyrylcholinesterase were added to each microwell and incubated for 5 min. The reaction was started by the addition of a substrate mixture, composed by equal parts of 3 mM 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB) and 75 mM acetylthiocholine iodide (ATChI) for AChE or butyrylthiocholine iodide (BuTChI) for BuChE. The absorbance at 415 nm was read at 0, 150, 300, and 450 s in a Bio-Rad Model 680 microplate reader (Bio-Rad Laboratories, USA). Enzyme inhibition was determined as the percentage activity of reaction media containing samples and the activity of control without inhibitor. All samples were tested in quadruplicate, the mean ± SD determined, and results expressed as IC$_{50}$ (μg mL^{-1}) and also as % inhibition at the highest concentration tested (150 μg mL^{-1}), since it allows comparison between extracts that did not achieve 50% inhibition but nonetheless present different activities.

In vitro cytotoxicity

The cytotoxicity against A549 human adenocarcinoma tumor cell line was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 96 wells plates were seeded with 20,000 A549 cells per well in

Dulbecco's modified Eagle's medium (DMEM), supplemented with 2% fetal bovine serum (FBS) and 1% penicillin–streptomycin mixture (10,000 UI mL^{-1}), in the presence of known concentrations of the extracts or standard previously dissolved in dimethyl sulfoxide (DMSO). The microplates were incubated for 48 h at 37 °C, 5% CO$_2$, and 98% humidity. Afterwards, 10 μL of 5 mg mL^{-1} MTT were added and the microplate was again incubated at 37 °C for 3 to 4 h. The culture medium was removed and DMSO was added to the wells to dissolve formazan crystals. The absorbance was read in a Bio-Rad Model 680 microplate reader at 550 nm. All samples were tested in quadruplicates, the mean ± SD determined and results were expressed as IC$_{50}$ (μg mL^{-1}) and also as % inhibition at the highest concentration tested (200 μg mL^{-1}), as referred above.

Spectroscopic analysis

ATR-FTIR spectra of the samples were obtained with a Perkin Elmer Spectrum Two, coupled with a Diamond ATR accessory (DurasamplIR II, Smiths Detection, UK). Thirty-two scans were acquired in transmittance mode in the range of 2000–650 cm^{-1}, with a wavenumber resolution of 1 cm^{-1}. All samples were analyzed in triplicate.

Results

Lipid screening

The amounts of total lipids from each algal species and lipid classes separated from the total extracts are presented in Table 1. Total lipids were higher in *Z. tournefortii* (9.66 ± 0.30 g (100 g)$^{-1}$ dw), followed by *A. taxiformis* (6.08 ± 0.45 g (100 g)$^{-1}$ dw) and *U. lactuca* (1.33 ± 0.03 g (100 g)$^{-1}$ dw). Neutral lipids and sterols were in a similar proportion in the *U. lactuca* and *A. taxiformis* extracts (50.63 ± 6.10 and 51.16 ± 4.85%, respectively) and in a lower proportion in *Z. tournefortii* (26.96 ± 8.22%). The glycolipids fraction was between 36.03 ± 8.08 and 16.11 ± 1.36% in *Z. tournefortii* and *U. lactuca*, respectively. The phospholipids fraction did not differ much between species, varying from 35.91 ± 2.36 to 31.60 ± 1.40% in *A. taxiformis* and *Z. tournefortii*, respectively.

TLC (thin layer chromatography) was used as a rapid and reliable way to ascertain biochemical differences between these three different macroalgae, screening the presence of neutral lipids (NL), glycolipids (GL), and phospholipids (PL) in the lipid content. TLC results are presented in Table 2 as a presence (+) or absence (−) of similar spots. Qualitative assessment of NL was carried out by comparison using cholesterol acetate, cholesterol, β-carotene, and phytol standards. The NL of *U. lactuca* and *Z. tournefortii* presented

Table 1 Total lipid content and class fractionation of *Ulva lactuca*, *Zonaria tournefortii*, and *Asparagopsis taxiformis*

Sample		Average ± standard deviation
Ulva lactuca	Total lipids	1.33 ± 0.03 g $(100$ g$)^{-1}$
(Chlorophyta)	Neutral and sterols	$50.63 \pm 6.10\%$
	Glycolipids	$16.11 \pm 1.36\%$
	Phospholipids	$35.84 \pm 3.40\%$
Asparagopsis taxiformis (Rhodophyta)	Total lipids	6.08 ± 0.45 g $(100$ g$)^{-1}$ dw
	Neutral and sterols	$51.16 \pm 4.85\%$
	Glycolipids	$23.68 \pm 1.92\%$
	Phospholipids	$35.91 \pm 2.36\%$
Zonaria tournefortii (Phaeophyta)	Total lipids	9.66 ± 0.30 g $(100$ g$)^{-1}$ dw
	Neutral and sterols	$26.96 \pm 8.22\%$
	Glycolipids	$36.03 \pm 8.08\%$
	Phospholipids	$31.60 \pm 1.40\%$

Data for total lipids are mean values ± standard deviation (SD) in g $(100$ g$)^{-1}$ of dry weight (dw). Lipid class fractionation (neutral and sterols, glycolipids, and phospholipids) are described as percentage (%) of total lipid content

spots with retention factors (R_f) similar to phytol. Unesterified sterols with an R_f similar to cholesterol was visible in all the macroalgae used in this work but esterified sterol and β-carothene were not detected. For GL fraction, a spot with the same R_f of digalactosyldiacylglycerol (DGDG) standard was found in *U. lactuca* but not on the other macroalgae. Concerning the phospholipid fraction, standards were L-α-phosphatidylcholine (PC), L-α-phosphatidylethanolamine (PE), 1-(3-*sn*-phosphatidyl)-*rac*-glycerol sodium salt (PG), 1,2-diacyl-*sn*-glycero-phospho-L-serine (PS), cardiolipin sodium salt (CL), and L-α-lysophosphatidylcholine (LPC). *Ulva lactuca* presented a similar R_f developed by CL and PC. *Asparagopsis taxiformis* and *Z. tournefortii* demonstrated

similar R_f with PE and PG standards. None of the macroalgae had similar R_f developed by PS and LPC standards.

Fatty acids

The results concerning fatty acids (FA) with carbon chain from C13 to C24 are presented in Table 3. Maximum value of total FA content was determined in *Z. tournefortii* (12.32 ± 1.28 mg g^{-1}), followed by *U. lactuca* (7.54 ± 0.92 mg g^{-1}) and *A. taxiformis* (6.01 ± 0.70 mg g^{-1}). Total SFA were higher in *A. taxiformis* (5.65 ± 0.66 mg g^{-1}), comprising about 94% of total FA, and the remaining being monounsaturated FA (MUFA). The majority of the SFA determined in *A. taxiformis* were palmitic (16:0) and myristic acid (14:0),

Table 2 Thin layer chromatography (TLC) performed in *Ulva lactuca*, *Zonaria tournefortii*, and *Asparagopsis taxiformis*

	Standards	*U. lactuca*	*A. taxiformis*	*Z. tournefortii*
Neutral lipids	Cholesterol acetate	−	−	−
	Cholesterol	+	+	+
	β-carothene	−	−	−
	Phytol	+	−	+
Glycolipids	DGDG	+	−	−
Phospholipids	PC	+	−	−
	PE	−	+	+
	PG	−	+	+
	PS	−	−	−
	CL	+	−	−
	LPC	−	−	−

Lipid classes comprising the lipid extract performed according to Folch et al. (1957) methodology, using the LE (lipophilic extract) was performed with CHCl$_3$:MeOH (2:1). The table indicates the presence (+) or absence (−) of similar retention factor (R_f) spots of the working standards for each lipid classes. Digalactosyldiacylglycerol (DGDG); L-α-phosphatidylcholine (PC); L-α-phosphatidylethanolamine (PE); 1-(3-sn-phosphatidyl)-rac-glycerol sodium salt (PG); 1,2-diacyl-sn-glycero-phospho-L-serine (PS); cardiolipin sodium salt (CL); L-α-lysophosphatidylcholine (LPC)

Table 3 Fatty acid composition performed by GC-MS in *Ulva lactuca*, *Zonaria tournefortii*, and *Asparagopsis taxiformis*

|	U. lactuca		Z. tournefortii		A. taxiformis	
	mg g^{-1}	% of TFA	mg g^{-1}	% of TFA	mg g^{-1}	% of TFA
14:00	0.07 ± 0.01	0.95 ± 0.02	0.76 ± 0.08	6.14 ± 0.20	1.14 ± 0.12	18.82 ± 0.18
16:00	3.76 ± 0.41	49.84 ± 1.00	3.97 ± 0.47	32.18 ± 0.57	4.32 ± 0.52	71.86 ± 0.25
18:00	0.07 ± 0	0.86 ± 0.02	0.09 ± 0.01	0.70 ± 0.03	0.14 ± 0.02	2.30 ± 0.02
16:01	0.41 ± 0.04	5.40 ± 0.04	3.30 ± 0.39	26.77 ± 0.47	0.04 ± 0.01	0.72 ± 0.02
18:01	1.86 ± 0.16	24.65 ± 0.07	2.03 ± 0.22	16.47 ± 0.29	0.32 ± 0.04	5.29 ± 0.07
18:2ω6	0.32 ± 0.02	4.23 ± 0.07	n.d.	n.d.	n.d.	n.d.
18:3ω3	0.43 ± 0.01	5.73 ± 0.35	n.d.	n.d.	n.d.	n.d.
18:4ω3	0.35 ± 0.02	4.60 ± 0.11	0.65 ± 0.06	5.24 ± 0.14	n.d.	n.d.
20:4ω6 (AA)	n.d.	n.d.	0.14 ± 0.01	1.17 ± 0.11	n.d.	n.d.
20:5ω3 (EPA)	n.d.	n.d.	0.32 ± 0.01	2.59 ± 0.28	n.d.	n.d.
22:5ω3	0.08 ± 0.01	1.11 ± 0.20	0.15 ± 0.01	1.23 ± 0.18	n.d.	n.d.
ω3	0.86 ± 0.03	11.44 ± 0.66	1.11 ± 0.06	9.05 ± 0.55	n.d.	n.d.
ω6	0.32 ± 0.02	4.23 ± 0.07	0.14 ± 0.01	1.17 ± 0.11	n.d.	n.d.
SFA	4.09 ± 0.42	54.28 ± 0.70	4.95 ± 0.57	40.13 ± 0.64	5.65 ± 0.66	94.00 ± 0.06
MUFA	2.26 ± 0.20	30.05 ± 0.08	5.33 ± 0.61	43.24 ± 0.43	0.36 ± 0.04	6.00 ± 0.006
PUFA	1.18 ± 0.05	15.67 ± 0.72	2.04 ± 0.14	16.63 ± 0.94	n.d.	n.d.
ω3 HUFA	0.08 ± 0.01	1.11 ± 0.20	0.47 ± 0.02	3.81 ± 0.45	n.d.	n.d.
Total mg g^{-1}	7.54 ± 0.92	–	12.32 ± 1.28	–	6.01 ± 0.70	–
ω6/ω3 Ratio	0.71		0.13		n.d.	
IT	0.59		0.35		30.02	
IA	1.18		1.06		24.57	

Data presented are mean values ± standard deviation (SD) of FAME in mg g^{-1} of dry weight (DW) or in percentage (%) of total fatty acids (TFA). *AA* arachidonic acid, *EPA* eicosapentaenoic acid, *ω* omega, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *HUFA* high unsaturated fatty acids, *n.d.* not detected, *IT* index of thrombogenicity, *IA* index of atherogenicity

accounting for almost 72 and 19% of total FA in this macroalgae. For *Z. tournefortii*, of the 12.32 ± 1.28 mg g^{-1} of FA, approximately 40% were SFA, 43% MUFA, and 17% PUFA. As above, the majority of SFA were palmitic and myristic acid, 32 and 6% of total FA, respectively. The MUFA were mainly represented by monounsaturated palmitic (16:1) and stearic acid (18:1), accounting for almost 27 and 17% of total FA, respectively. Concerning the polyunsaturated ω3 and ω6 FA, these were mainly represented by stearidonic (18:4ω3), eicosapentaenoic (20:5ω3), docosapentaenoic (22:5ω3), and arachidonic (20:4ω6) acids, representing 5, 3 for the former and 1% for the last two PUFA, respectively. For *U. lactuca*, which contained 7.54 ± 0.92 mg g^{-1} of FA, approximately 54% were SFA, 30% MUFA, and 16% PUFA. Again, palmitic acid represented the majority of SFA, accounting for 50% of total SFA in *U. lactuca*. The MUFA were mainly represented by the monounsaturated stearic (25%) and palmitic acid (5%). PUFA in *U. lactuca* were characterized primarily by α-linolenic, stearidonic, linoleic, and docosapentaenoic acids, representing 6, 5, 4, and 1%, respectively, of total FA in this macroalgae. The ω6/ω3 ratio was determined and higher values were achieved in *U. lactuca*

(0.71) and *Z. tournefortii* (0.13). It was not possible to calculate this ratio for *A. taxiformis*, due to undetermined values for PUFA.

ATR-FTIR analysis

The three macroalgae studied in this work were assessed using ATR-FTIR analysis (Fig. 1), for comparison between them and to determine the peaks that characterize each of the species. Sixteen important bands were detected from 650 to 2000 cm^{-1}, the transmittance transformed to absorbance and a comparison was carried out. At 790 cm^{-1} (A) and 846 cm^{-1} (B), two small peaks were distinguished which could represent the C–O–S vibration in the equatorial position and axial position, respectively. A strong IR band (C) at 1020 cm^{-1} was detected, indicating a C–O–C bending of polysaccharides constituents. Another peak was detected at 1050 cm^{-1} (D), representative of C–O–C skeletal vibration and at 1080 cm^{-1} (E) a vibration of C–O–C structure of polysaccharides. Likewise, a shoulder at 1161 cm^{-1} (F) was detected, corresponding to vibration of C–O–C of carbohydrates from polysaccharides. Furthermore, at 1222 cm^{-1} (G), a medium-
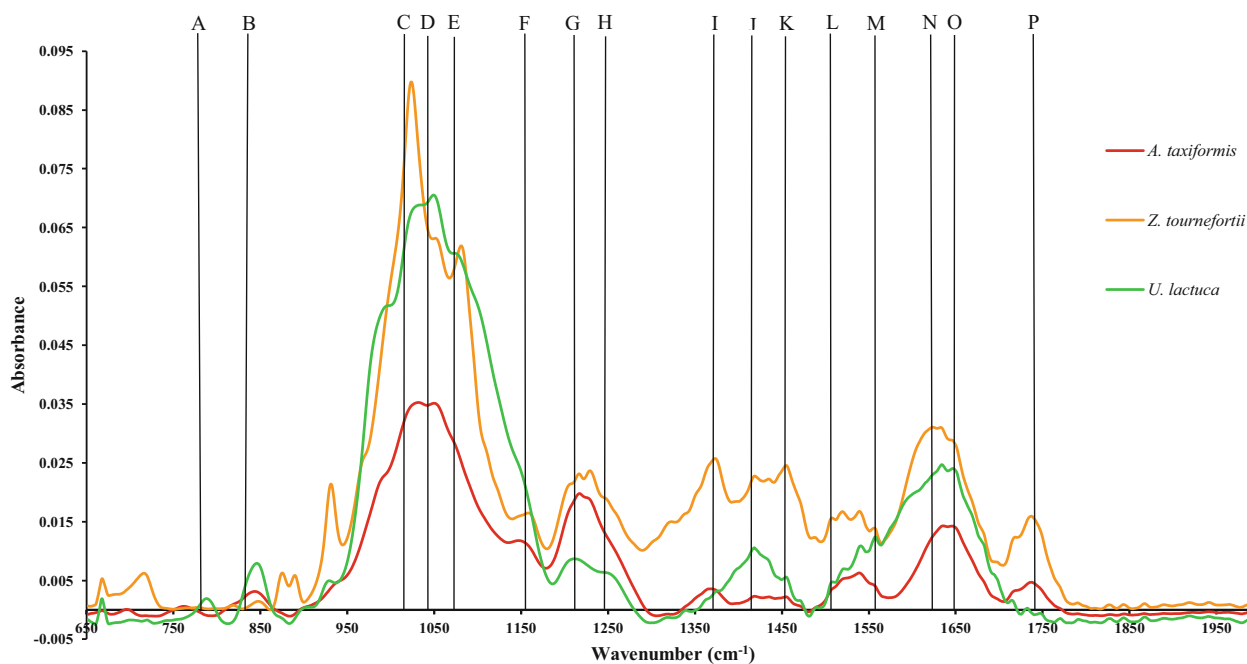
Fig. 1 ATR-FTIR spectra, plotting wavenumber (cm^{-1}) by absorbance, for the macroalgae lyophilized with bands from A to P indicating important peaks

intensity S=O vibration arouse and a shoulder at 1256 cm^{-1} (H) which indicates the asymmetric O=S=O stretching vibration of sulphate esters with possibly some contribution of COH, CC, and CO vibrations. A medium-intensity peak was visualized at 1375 cm^{-1} (I), representative of symmetric deformations in CH$_2$ in proteins and N (CH$_3$)$_3$ methyl bending in lipids. At 1434 cm^{-1} (J), a C–H bending vibration was detected and at 1455 cm^{-1} (K), a scissoring vibration of CH$_2$ or/and an asymmetric bending vibration of CH$_3$ of fucose. Between 1510 cm^{-1} (L) and 1558 cm^{-1} (M), four peaks emerged and their presence is attributed to bending vibrations of N-H of protein amide II and stretching vibrations of C-N. Similarly, medium-intensity peaks were identified at 1622 cm^{-1} (N) and 1648 cm^{-1} (O), representing the stretching vibrations of C=O of protein amide I. At 1739 cm^{-1} (P), a small intensity peak was observed which corresponds to C=O vibration of aldehyde.

Bioactivity

The anti-acetylcholinesterase (anti-AChE) and anti-butyrylcholinesterase (anti-BuChE) activities for the lipophilic extract (LE) extracts and the ethanolic extracts (EE) are presented in Table 4. These extractions were compared to determine the difference in bioactivity when using extracts performed using chloroform and methanol (LE) or a scalable extractor (Timatic) that can be used in the industry with a more environmental friendly solvent, ethanol (EE). The results have shown that LE extracts were more active AChE inhibitors when compared with EE, as seen by the lower IC$_{50}$ values and/or higher inhibition percentages at 150 μg mL^{-1}. The highest activity was found in *A. taxiformis* extracts, with IC$_{50}$ = 8.92 ± 0.43 and 46.33 ± 6.02 μg mL^{-1} for LE and EE, respectively. Concerning BuChE inhibition, once again, *A. taxiformis* was more active, with 13.96 ± 0.32 and 28.1 μg mL^{-1} for LE and EE, respectively. All of these extracts were double cholinesterase inhibitors, except for *U. lactuca* EE, and the high activity of *A. taxiformis* LE must be highlighted, with low IC$_{50}$ values and in the case of BuChE inhibition, almost four-fold more active than Donepezil standard, which is used in the treatment of Alzheimer's disease.

The cytotoxicity in A549 adenocarcinomic human alveolar basal epithelial cells was also assessed, with results presented in Table 5. The IC$_{50}$ results ranged between 98.02 ± 0.23 and 140.42 ± 4.99 μg mL^{-1}, for *A. taxiformis* LE and EE extracts, respectively.

Discussion

Total lipids

The macroalgae analyzed in this work have a low lipid content, which is in agreement with previous works (Paiva et al.

Table 4 In vitro anti-cholinesterasic potential of macroalgae extracts

	AChE activity		BuChE activity	
	% inhibition (150 µg mL^{-1})	IC$_{50}$ (µg mL^{-1})	% inhibition (150 µg mL^{-1})	IC$_{50}$ (µg mL^{-1})
U. lactuca LE	37.06 ± 1.06	> 150	16.52 ± 4.72	> 150
U. lactuca EE	0	> 150	0	> 150
Z. tournefortii LE	42.73 ± 1.70	> 150	19.59 ± 5.55	> 150
Z. tournefortii EE	40.12 ± 4.61	> 150	30.81 ± 3.26	> 150
A. taxiformis LE	100	8.92 ± 0.43	100	13.96 ± 0.32
A. taxiformis EE	100	46.33 ± 6.02	100	28.10 ± 0.93
Donepezil	95.20 ± 0.40	0.01 ± 0.00	100	55.62 ± 3.47
Galantamine	98.40 ± 1.50	0.43 ± 0.09	37.80 ± 0.90	> 150

For the percentage (%) of inhibition using the concentration of 150 µg mL^{-1} of extract, data is presented as %. The IC$_{50}$ results are presented as the concentration of extract needed, in µg mL^{-1}, to inhibit 50% of the enzyme. LE (lipophilic extract) indicates that extract was performed with CHCl$_3$:MeOH (2:1). EE (ethanolic extract) indicates that the extract was performed with Timatic extractor using 96% ethanol. *AChE* acetylcholinesterase, *BuChE* butyrylcholinesterase

2014; Kendel et al. 2015). *Ulva lactuca* lipid content was 1.33 ± 0.03 g (100 g)$^{-1}$ dw, which is in the middle of the values range reported by Santos et al. (2015), who obtained 0.3 g (100 g)$^{-1}$ dw *U. lactuca* produced by aquaculture, and by Khotimchenko et al. (2002), who reported 3.06 g (100 g)$^{-1}$ dw in *U. lactuca* collected in Bodega Bay, California. Therefore, lipid content can be extremely variable in this species, possibly according to environmental differences. Ragonese et al. (2014) also evaluated *A. taxiformis* collected in the Sicilian coast and determined a lipid yield of 12.22 g (100 g)$^{-1}$ dw, roughly twice that we have determined (6.08 ± 0.45 g (100 g)$^{-1}$ dw). The same author analyzed *Dictyota dichotoma* and the lipid content was found to be less than *Z. tournefortii*, belonging to the same family, these macroalgae contain 6.27 and 9.66 ± 0.30 g (100 g)$^{-1}$ dw, respectively. Burreson et al. (1976) determined 42 different components in the essential oil of *A. taxiformis*, collected in

Hawaii, and the bromoform CHBr$_3$, used in the past as a sedative, is the major constituent. It was also stated that although being the favorite edible macroalgae in Hawaii archipelago, the existence of haloforms and halogenated compounds in its essential oil could mean that this macroalgae could be poisonous when consumed. It was not possible to compare results concerning lipid classes with other published works, since few reports exist assessing these lipid groups in these macroalgae. The most similar work is that of Kendel et al. (2015), who evaluated *U. armoricana* and determined that NL was 55.60% (ours 50.63%), GL was 29.10% (ours 16.11%), and PL is 15.30% (ours 35.11%). Comparing these results, we can determine that these lipid classes yield varies significantly, even for macroalgae of the same genus.

The TLC analysis performed for each lipid class revealed that *U. lactuca* lipid composition could contain phytol and a type of cholesterol compound in its NL also revealed the same spots R_f as DGDG in its GL and developed similar R_f to cardiolipin and L-α-phosphatidylcholine in its PL. Phytol is a remarkable compound due to its use as a precursor of industrial synthesis of vitamins E and K (Kendel et al. 2015). The *Z. tournefortti* also revealed in its NL classes similar R_f presented by phytol and cholesterol and the PL classes contain similar R_f presented by L-α-phosphatidylethanolamine and 1-(3-sn-phosphatidyl)-*rac*-glycerol. The *A. taxiformis* revealed similar spots with an R_f similar to cholesterol in its NL fraction and spots with similar R_f presented by phospholipids in *Z. tournefortii* PL fraction. Ragonese et al. (2014) assessed these classes in 8 macroalgae (2 green, 2 red, and 4 brown) and determined that *U. rigida* revealed also analogous spots with an R_f similar to L-α-phosphatidylcholine. The same author determined that some species of red and brown macroalgae also present spots with the same R_f as presented by L-α-phosphatidylethanolamine and 1-(3-sn-phosphatidyl)-*rac*-glycerol.

Table 5 Cytotoxicity effect of macroalgae extracts against A549 tumor cell line

	% toxicity (200 µg mL^{-1})	IC$_{50}$ (µg mL^{-1})
U. lactuca LE	0	> 200
U. lactuca EE	90.15 ± 6.96	140.42 ± 4.99
Z. tournefortii LE	n.d.	n.d.
Z. tournefortii EE	90.41 ± 1.52	137.64 ± 7.89
A. taxiformis LE	100	98.02 ± 0.23
A. taxiformis EE	20.14 ± 5.65	> 200
Colchicine	71.69 ± 0.96	2.78 ± 0.71
Paclitaxel	97.44 ± 0.66	5.96 ± 0.98

The IC$_{50}$ results are presented as the concentration of extract needed, in µg mL^{-1}, to inhibit tumor cell viability by 50%. LE (lipophilic extract) performed with CHCl$_3$:MeOH (2:1). EE (ethanolic extract) performed with Timatic extractor using 96% ethanol. *n.d.* not determined

Fatty acids

The FA yield in this work was higher in *Z. tournefortii* (1.23% dw), followed by *U. lactuca* (0.75% dw) and *A. taxiformis* (0.6% dw). Schmid et al. (2018) evaluated 61 species of macroalgae collected along the Southern Australian shore and also determined the TFA for the *Dictyopteris muelleri*, from the same family that *Z. tournefortii* (Dictyotaceae). The TFA varied from 1.6 to 4.8%, for *Z. turneriana* and *D. muelleri*, respectively. This author also determined for *Ulva* a variation between 0.5 to 1.9% of TFA and for the *Ptilonia australasica* and *Delisea pulchra*, belonging to the same family as *A. taxiformis* (Bonnemaisoniaceae), 0.9% and 2.2%, respectively. Our values agree with or were close to the reported values, with some discrepancy due to environmental factors such as availability of nutrients in seawater, light availability, herbivory pressure, seasonal period of harvest, and geographical location.

Highest amount of SFA was detected in the red alga *A. taxiformis*, 94% of total FA of which 72% is comprised by palmitic acid (C16:0). This result does not agree with of Mellouk et al. (2017), who reported 23.7% of SFA and 67.8% of unsaturated fatty acids (UFA) for the same species, from the Mediterranean, near the Algerian coast. This enormous difference is not easy to explain, although it is probably due to environmental differences between the Mediterranean and the open Atlantic Ocean, such as water temperature and hydrodynamism. It also raises the need to assess nutritional profiles of macroalgae collected in different locations and possibly also at different phases of the life cycle. Gressler et al. (2010) determined that SFA varied from 51 to 78% and palmitic acid is the most representative SFA in four species of red macroalgae, collected in the intertidal zone of Anchieta, Espírito Santo State, Brazil. SFA composition of *Z. tournefortii* (40%) and *D. muelleri* (39%) was similar (Schmid et al. 2018). The FA composition of the chlorophyte *U. lactuca* subjected to different environments was analyzed. For instance, Cardoso et al. (2017) evaluated this macroalgae cultivated in fish ponds and demonstrated that SFA were lower (38%) when comparing with our results (54%) with palmitic acid (16:0) accounting for 19% and 50% of total FA, respectively. Mai et al. (1994) assessed the nutritional quality of *U. lactuca* for the nutrition of two species of abalone pointing out that palmitic acid (24%) is a major component of FA fraction in *U. lactuca*. The assessment of *U. lactuca* collected by Verma et al. (2017) from the intertidal zone, in Port Okha (Gujarat), India, also revealed high yield of this SFA (31%). Kendel et al. (2015) evaluated two macroalgae, *Ulva armoricana* and *Solieria chordalis*, from Brittany (France) and determined that palmitic acid was predominant in these macroalgae. Furthermore, Sánchez-Machado et al. (2004) evaluated 5 edible macroalgae, *Himanthalia elongata*, *Laminaria ochroleuca*, *Undaria pinnatifida*, *Palmaria* sp.,

and *Porphyra* sp., along the northwest Iberian Coast, whereas the SFA varies from 20.39 to 64.95%, and palmitic acid being again the major FA, with values between 16.51 until 63.19% of total FA.

The highest value of MUFA was detected in the *Z. tournefortii* (43.27%), followed by *U. lactuca* (30.04%) and *A. taxiformis* (6.01%). The most prominent FA among MUFA changed between species, since in *U. lactuca* and *A. taxiformis* was the stearic acid, with 24.65 and 5.29%, respectively, whilst in *Z. tournefortii*, the major concentration of MUFA was palmitic acid, corresponding to 26.80% of total FA. Cardoso et al. (2017) determined to be the most representative MUFA (16.6%) in *U. lactuca*, collected from fish ponds, the stearic acid with 7.7% of total FA. Dawczynski et al. (2007) determined that MUFA content varies between 7.8 and 20.7% in 4 dried edible macroalgae *Porphyra* sp., *U. pinnatifida*, *Laminaria* sp., and *Halymenia fusiforme*, with stearic acid being the most representative, varying from 5.95 to 15.3% of total FA.

The highest content of PUFA was determined in *Z. tournefortii*, 2.04 ± 0.14 mg g^{-1} dw (16.58% of TFA), followed by *U. lactuca* with 1.18 ± 0.05 mg g^{-1} dw (15.62% of TFA), whilst no PUFA was detected in *A. taxiformis*. Sánchez-Machado et al. (2004) determined that PUFA content varies between 16.10 and 69.11% in edible macroalgae, with our values close to the lowest assessed content. The most representative PUFA was determined to be species dependent. In *U. lactuca*, the α-linolenic acid, 0.43 ± 0.01 mg g^{-1} dw (5.71% of TFA), and *Z. tournefortii* the stearidonic acid, 0.65 ± 0.06 mg g^{-1} dw (5.24% of TFA), are the main PUFA components. According to several scientific reports, the most representative PUFA in seaweed appears to be variable. Cardoso et al. (2017) and Wahbeh (1997) refer that linoleic acid is the principal PUFA in *U. lactuca*, composing 9.5 and 9.7%, respectively. On the other hand, Mai et al. (1994) determined that stearidonic acid was the most representative PUFA in *U. lactuca* (14.8%). Verma et al. (2017), studying this macroalgae, determined that α-linolenic acid was the major PUFA, coinciding with our finding, but with higher 14.31% in the TFA. The eicosanoid precursor, arachidonic acid, 0.14 ± 0.01 mg g^{-1} dw (1.16% of TFA), and eicosapentaenoic acid, having 0.32 ± 0.01 mg g^{-1} dw (2.57% of TFA), were only determined in *Z. tournefortii*. Sánchez-Machado et al. (2004) also demonstrated that the most representative PUFA is also macroalgae dependent, with arachidonic acid having the highest content in *Porphyra* sp. (6.80%), *H. elongata* (10.69%), and *L. ochroleuca* (14.20%). Stearidonic acid is present in *U. pinnatifida* with 22.60% and the eicosapentaenoic acid in E *Palmaria* sp. with 24.05%. These different observations allow us to conclude that the major macroalgae FA content is seasonal dependent, and will vary depending on the geographical location and ecosystem dynamics. This conclusion is supported by dependent Nelson et al. (2002) and Polat and Ozogul (2013).

The ω6/ω3 ratio in *U. lactuca* was 0.71 and in *Z. tournefortii* 0.13, with no amount of PUFA detected in *A. taxiformis*. Sánchez-Machado et al. (2004) determined that this ratio varies from 0.13 to 1.21 in 5 edible macroalgae, Cardoso et al. (2017) determined a 0.86 ratio for *U. lactuca* grown in fish aquaculture ponds and Schmid et al. (2018) establish a 0.8 ratio for *Z. turneriana* collected in the Australian southern shore. It is currently accepted that human beings evolved with a ω6/ω3 ratio diet of approximately 1, but nowadays the western diets are between 15/1 and 16.7/1 ratios, whereas a maximum of 4/1 ratio is considered suitable to maintain a healthy status (Simopoulos 2002).. Elevated ω6/ω3 ratio increase the prostanoid thromboxane (TXA_2) and inhibit prostacyclin (PGI_2) production which is linked to the increasing risk of thrombosis (Ulbricht and Southgate 1991). Moreover, Simopoulos (2002) described in his work that a ω6 rich diet could increase blood viscosity, vasospasm, and vasoconstriction, when a higher consumption of ω3 helps the relaxation of large arteries and vessels, due to an increase of nitric oxide, an endothelium-derived relaxing factor (EDRF), thus decreasing the atherogenicity effect. Ulbricht and Southgate (1991) developed two interesting equations, one for calculating an index of thrombogenicity (IT) and an index of atherogenicity (IA), where values indicate healthier results. For the macroalgae included in this work, the IT was 0.35 for *Z. tournefortii*, 0.59 for *U. lactuca*, and 30.02 for *A. taxiformis*. These results indicate an anti-thrombogenic effect of *Z. tournefortii* and *U. lactuca*, but due to the absence of PUFA in *A. taxiformis*, this macroalga appears unsuitable for this purpose. In addition, the toxicity of halogenated compounds in this alga should be pointed out (e.g., Li et al. 2016). For IA, the values were 1.06 for *Z. tournefortii*, 1.18 for *U. lactuca*, and 24.57 for *A. taxiformis*. These results evidence an anti-atherogenicity effect of *Z. tournefortii* and *U. lactuca*, whilst *A. taxiformis* is confirmed as unsuitable for this purpose. Vizetto-Duarte et al. (2015) determined these indices in six different *Cystoseira* species and determined IT values varying between 0.54 and 1.61, and IA between 0.67 and 1.94. Belattmania et al. (2018) also analyzed brown macroalgae from Morocco and determined IT to vary between 0.04 to 0.25 and IA from 0.55 to 1.35.

These results strongly enforce the possibility of using *U. lactuca* and *Z. tournefortii* lipids to enhance the nutraceutical characteristics of food products and to develop macroalgae-based supplements to improve or maintain health. Considering the above, including *U. lactuca* and *Z. tournefortii* in human nutrition could be a means to reduce the ω6/ω3 ratio.

ATR-FTIR

This technique is extensively used for the recognition and characterization of numerous types of molecules, using the fact that it delivers an exclusive signature to each compound.

The transmittance variance detected by the equipment in each wavenumber is due to the relative mass and geometry of the atoms in the sample. The conformation of the molecules enables resonance between vibrations that will modulate the spectra (Derenne et al. 2014). The assessment of our samples allowed distinguishable ATR-FTIR "fingerprints" to be obtained, and two small peaks, most prominent in *U. lactuca*, with 790 cm^{-1} (A) and 846 cm^{-1} (B), according to Robic et al. (2009), indicate the presence of ulvans. These complex polysaccharides are integrated in the cell wall and due to their unusual composition and structure considered a functional biopolymer (Lahaye and Robic 2007). Three well-distinguished peaks and one shoulder located between 1000 and 1200 cm^{-1} (C, D, E, and F) indicate the vibration of sugar rings, overlapping with stretching vibrations of C–OH side groups and the C–O–C glycosides bonds. According to Robic et al. (2009), ulvan extracts present strong absorption at 1055 cm^{-1}, close to our D peak at 1050 cm^{-1}. This is well distinguished for *U. lactuca*, due to C–O stretching in rhamnose and glucuronic acid. A medium-intensity peak at 1222 cm^{-1} (G) and 1256 cm^{-1} (H) was detected in the three macroalgae, indicating polysaccharides with some degree of sulfation. Generally, sample sulfation intensity is detected between 1210 and 1260 cm^{-1} (Gómez-Ordóñez and Rupérez 2011). In green macroalgae this would be represented by ulvans, in red macroalgae by carrageenans, and for brown macroalgae by fucoidan. These polysaccharides are valuable for their intrinsic rheological qualities (carrageenans) or for their bioactive properties (ulvans and fucoidan). For example, fucoidan, a fucose-enriched sulfated polysaccharide with a high degree of complexity, is described as a compound with anti-inflammatory, anticoagulant, antithrombotic, and antioxidant properties (Senthilkumar et al. 2013) and is exploited as an additive in some products for health food, drinks, and cosmetics (Pádua et al. 2015). These biological properties will vary, depending on the density and specific positions of these sulfated groups (Berteau and Mulloy 2003).

Bioactivity

Anti-acetylcholinesterasic activity was higher for LE when compared with EE, which can be seen by the lower IC$_{50}$ results and/or the higher % inhibition at the highest concentration tested. These results suggest that the molecules responsible for this effect are either lipids or similar compounds. The macroalgae *A. taxiformis* stands out, presenting 100% inhibition using 150 μg mL^{-1} for both types of extract and the lowest IC$_{50}$ results (8.92 ± 0.43 μg mL^{-1}) using LE extract. The EE of this macroalgae also delivered good results, with an IC$_{50}$ of 46.33 ± 6.02 μg mL^{-1}. For comparison, two widely used drugs in Alzheimer's patients were used in this work: donepezil, a piperidine derivative, and galantamine, an alkaloid. Although the activity shown by these compounds was

much stronger, it must be pointed out that the activities of the LE and EE *A. taxiformis* extracts, which are mixtures containing a high variety of compounds, are nonetheless high enough to justify a purification to identify the active molecules. These results are far higher than those presented by Stirk et al. (2007), who evaluated 7 macroalgae species from the east coast of South Africa determining the IC_{50} results for AChE inhibitory activity. The best result obtained by those authors (IC_{50} of 4800 μg mL^{-1} for *Dictyota humifusa*) is more than 500 times less active than the *A. taxiformis* EE presented herein. The most interesting result reported by Stirk et al. (2007) is the effect of seasonality on the activity, which may be due to differences in environmental pressures such as predation along the year cycle. The work of these authors point out the need to carry out studies determining the best phase of the algal cycle to collect biomass with significant bioactivity.

Most of the extracts tested also inhibited butyrylcholinesterase, and once again *A. taxiformis* was most prominent, with LE approximately two-fold more active than EE. In addition, the dual inhibitory activity of AChE and BuChE is quite positive, since there is evidence that compounds with this feature can intensify the effectiveness of AD treatment, possibly by also reducing the formation of β-amyloid plaques (Giacobini 2003).

Overall, the results obtained further reinforce the potential of *A. taxiformis* extracts for further studies to inhibit these enzymes, since they are much stronger than results reported by other authors for macroalgae extracts. *Cystoseira* species extracts (Custódio et al. 2016) have a dual AChE and BuChe inhibition, but at much higher concentrations (between 1000 and 10,000 μg mL^{-1}) than the ones used herein (0.293 and 150 μg mL^{-1}). What is mentioned above for AChE inhibition is also true for BuChE, i.e., that the compounds with higher anti-cholinesterasic activity are probably lipids or compounds with a similar polarity, since LE extracts were consistently better inhibitors of both enzymes. To avoid the use of chloroform, the ethanolic extraction (EE) could be optimized to effectively extract these compounds, varying the time and number of compression and decompression cycles. These parameters enable the variable pressure for solvent recirculation through the sample. Solvent mixtures could also be applied, adding ethyl acetate, an extensively used solvent in the food industry to decrease polarity. This effort is necessary to develop "greener" methodologies to produce extracts comprising of apolar extracts that could perform equally, efficient reducing the usage of toxic solvents, known to be harmful to the environment.

The cytotoxicity against A549 tumor cell line does not present a clear activity pattern as in the case of anti-cholinesterasic activities. The *A. taxiformis* LE, *Z. tournefortii* EE, and *U. lactuca* EE were the most active extracts, with IC_{50} values of 98.02 ± 0.23, 137.64 ± 7.89, and 140.42 ± 4.99 μg mL^{-1}, respectively. *Asparagopsis taxiformis* LE again exhibited the best results, highlighting the bioactive potential of this macroalgae and extract for further compound

purification. These values are comparable with others reports of activity against this cell line, such as the IC_{50} of 110 μg mL^{-1} obtained by Dellai et al. (2013), using *Laurencia obtusa* MeOH:CHCl$_3$ extracts. Kang et al. (2017) obtain an IC_{50} of 50 μg mL^{-1} against A549 cells, inducing apoptosis, using *Gracilariopsis lemaneiformis*. However, this result corresponds to purified polysaccharides and is therefore not comparable with the extracts presented here, since they were extracted with distilled water. Additionally, an *Ecklonia cava* ethyl acetate extract displayed in vitro metastatic activity against A549 cells, although it did not affect cell viability (Lee et al. 2011). These results suggest that aqueous and fractions of intermediate polarity may also have anticancer potential and should also be investigated. Concerning the results presented herein, the extracts and/or isolated compounds isolated thereof will be tested against other cell lines, namely obtained from non-tumor origin, and the mechanism of toxicity will be assessed. However, in a preliminary study, cytotoxicity against a cell line is a good indicator of potential for further studies using a bioassay guided approach.

Conclusion

The macroalgae analyzed in the present work present interesting characteristics that justify further research. *Ulva lactuca* and *Z. tournefortii* have qualities that point out their interest in nutrition, such as their low lipid content and favorable IT and IA values. The presence of polysaccharides such as ulvans or fucoidans with some degree of sulfation, as indicated by ATR-FTIR results, also points out the interest of these two macroalgae as staple food, if not for human consumption, a least in animal feed. Concerning *A. taxiformis*, the high percentage of SFA and the extremely high IA and IT values and other studies which point out to the toxicity of its halogenated compounds do not recommend it as an edible macroalgae, but it displays biological activities that indicate its pharmaceutical interest. The low IC_{50} values for anti-cholinesterasic activity, particularly those for the LE extract, and the fact that both *A. taxiformis* extracts present dual AChE and BuChE inhibition, are good indicators of the possibility of finding a good candidate scaffold for an anti-Alzheimer Disease drug.

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