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Undaria pinnatifida (*U. pinnatifida*) bioactivity: Antioxidant, gastro-intestinal motility, cholesterol biosynthesis and liver cell lines proteome

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ABSTRACT

Undaria pinnatifida is a brown alga, largely used as food in oriental countries. The purpose of this work was to analyze several bioactivities that might be present in different food preparations, relating them with the compounds present in the extracts. Aqueous extracts cooked, like a soup, as well as used in a salad, by simulating *U. pinnatifida* digestion with pancreatin were prepared. The soup preparation had anti-acetylcholinesterase activity as well as 3-hydroxy3-methyl-glutaryl coenzyme A reductase inhibition capacity, the use of 1 mg/mL, for both enzymes, caused 50% enzyme activity inhibition. The extracts produced changes in HepG2 cell proteome, mainly in the 148 kDa proteins. *U. pinnatifida* in salads hardly released phlorotannins to the digestive tract. The *in vitro* studies suggested that a soup prepared from this seaweed could deliver bioactive compounds, phlorotannins and small peptides that may facilitate the gastro-intestinal motility and reduce the cholesterol biosynthesis.

1. Introduction

Algae have a long tradition of being consumed in Japan. An Imperial law in 701 AC dictated the payment of taxes in algae, among which *Undaria* sp can be referred (Nisizawa, Noda, Kikuchi, & Watanabe, 1987). In the 10th century, twenty one types of algae were already known and used as food in Japanese cuisine (Nisizawa et al., 1987). Macroalgae, or seaweeds, have a long tradition of human consumption in other parts of the world. Archaeological remains, dating back 14000 years, were also found in Chile (Wells et al., 2017). *U. pinnatifida* (Harvey) Suringar, also known as wakame, became a species of considerable economic importance as food source in Japan, Korea and China (Guiry & Guiry, 2020).

U. pinnatifida was translocated to Atlantic France in 1971 (Pickering, Skelton, & Sulu, 2007). It acclimated, became invasive and now appears in Spain, Portugal and several Atlantic European countries (Epstein, Foggo, & Smale, 2019). European *U. pinnatifida* supply – which also includes another related brown seaweed, *Alaria esculenta* (Linnaeus) Greville – is mainly imported from Asia (56%), but Spain, France and the Netherlands are also producing for the internal market (Camia et al., 2018). Algae are claimed as being a healthy food with high content in protein and fiber and low content in fat (Taboada, Millán, & Miguez, 2013). *U. pinnatifida* also possesses a high content in minerals and vitamin A (Taboada et al., 2013). Besides these biomolecules, seaweeds also have secondary metabolites like phlorotannins (Wan-Loy & Siew-Moi, 2016) and small peptides (Daliri, Oh, & Lee, 2017; Lafarga, Acién-Fernández, & Garcia-Vaquero, 2020) with biological activities. Phlorotannins are compounds synthetized from phloroglucinol or 1,3,5-trihydroxybezene, obtained through the condensation of malonyl-CoA with acetyl-CoA. Phlorotannins have different chemical structures. They can have ether, phenyl or dibenzodioxin linkages (Li, Wijesekara, Li, & Kim, 2011). Brown algae may contain approximately 15% of phlorotannins in dry matter (Imbs & Zvyagintseva, 2018). Several biological activities have been attributed to these structures. These

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compounds can be antioxidant (Cuong, Boi, Van, & Hau, 2016) and enzyme inhibitors (André et al., 2020; Kannan, Aderogba, Ndhlala, Stirk, & Van Staden, 2013; Kim et al., 2019). They have also shown antitumoral (Yang, Zeng, Dong, Liu, & Li, 2010), antibacterial (Eom, Kim, & Kim, 2012) and anti-inflammatory activity (Dong et al., 2019). Several reviews have pointed out the bioactivities of *U. pinnatifida* (Epstein & Smale, 2017; Wang, Park, Jeon, & Ryu, 2018). Brown alga demonstrated reducing cholesterol levels in humans having hypercholesterolemia (Choi et al., 2015). The administration of dry *U. pinnatifida* to rodent diet caused a decrease in LDL-cholesterol in the laboratory animals (Taboada et al., 2013).

Bioactive small peptides have also shown several biological activities (Sánchez & Vázquez, 2017), acting as antioxidants and immunomodulators and having antihypertensive and cholesterol lowering effects (Turpeinen et al., 2009).

The mechanisms of decreasing cholesterol blood levels are complex and subject to cell regulating mechanism. The decrease of the cholesterol biosynthesis in the liver or the reduction of dietary cholesterol absorption can be referred for clinical use. Cholesterol biosynthesis decrease is done through the use of statins that inhibit 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the regulator enzyme in the cholesterol biosynthesis pathway (Endo, 2010). The reduction of dietary cholesterol absorption can be achieved by increasing the gastro-intestinal motility through the inhibition of the enzyme acetylcholinesterase (AChE) localized in the neuro-muscular junctions (Gašperšič, Koritnik, Črne-Finderle, & Sketelj, 1999).

The accumulation of cholesterol in the blood vessels can lead to atherosclerosis, an inflammatory disease that has a very high rate of mortality due to the origin of strokes or other heart problems (Marulanda-Londoño & Chaturvedi, 2016). Antioxidants can have a role in the prevention or even in the treatment of atherosclerosis either by avoiding the formation of atherosclerotic plaques or the formation of radical species that may produce lipoprotein oxidations (Malekmohammad, Sewell, & Rafieian-Kopaei, 2019). There has been a search for new natural antioxidants (Malekmohammad et al., 2019) and algae are also described as having high antioxidant activity (Balboa, Conde, Moure, Falqué, & Domínguez, 2013), meaning that they can be a good source of this type of compounds. Nevertheless, the consumption of seaweeds is not devoid of concern (Cherry, O'hara, Magee, Mcsorley, & Allsopp, 2019). Algae, besides having high levels of salt, iodine or other metals, are bioaccumulative organisms that absorb several compounds during their growth in the river's estuary, sometimes used for removal of pollutants from wastewaters (Arumugam et al., 2018; Sahu et al., 2020) and can also have several bacterial pathogens mainly if collected near coastal regions (Barberi, Byron, Burkholder, St. Gelais, & Williams, 2020).

The aim of the present work is to study the effects of *U. pinnatifida* aqueous extracts similar to a soup preparation or a salad consumption on antioxidant activity, cholesterol indirect reduction by increasing the gastro-intestinal motility and cholesterol direct reduction by HMGR inhibition. In addition, the effect of the extracts on liver cancer cells proteome is also studied with the focus on the identification of the secondary metabolites and small peptides involved in these bioactivities.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. During the analysis of the extracts by HPLC-DAD acetonitrile (ACN) from Carlo Erba (Peypin, France) and trifluoroacetic acid (TFA) from Merck (Darmstadt, Germany) were used and in mass spectrometry analysis the eluents used were ACN, formic acid and water from Fisher Scientific, Optima™ (Hampton, NH, USA). Gallic acid, Folin-Ciocalteu reagent, pancreatin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin, acetylcholinesterase (AChE), acetylcholine iodide (AChI), 5-5′-dithiobis(2-nitrobenzoic acid) (DTNB), galantamine, HMG-CoA Reductase assay kit (CS1090), trypan blue, 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium bromide (MTT), ammonium persulfate (PSA), glycine, iodoacetamide and sodium dodecyl sulfate (SDS) were obtained from Sigma (Barcelona, Spain). Sodium carbonate, sodium chloride, potassium dihydrogen phosphate, potassium hydrogen phosphate, tris(hydroxymethyl)aminomethane (Tris), sodium chloride, 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), glacial acetic acid were bought from Merck. Ethanol 96% was acquired to Carlo Erba, methanol to Riedel-de Haën (Seelze, Germany) and hydrochloric acid to Fisher (Waltham, MA, USA). Magnesium chloride hexahydrate and bromophenol blue were bought to PanReac (Barcelona, Spain) and RPMI medium, fetal bovine serum (FBS), pen-strep (penicillin 100 U/mL and streptomycin 100 U/mL), trypsin (10x) and L-glutamine (2 Mm) to Lonza (Basel, Switzerland). Protein marker NZYBlue and tetramethylethylenediamine (TEMED) were purchased from NZYTech (Lisbon, Portugal), glycerol and agarose from Promega (Wisconsin, USA), Acrylamide/bis (29: 1) from Amresco (Framingham, MA, EUA), Coomassie Brilliant Blue R-250 from Bio-Rad (Hercules, CA, USA) and dithiothreitol (DTT), immobilized pH gradient buffer pH 3–11 NL (IPG), urea, thiourea and 2-D Quant kit (80-6483-56) from GE Healthcare Life Sciences (Marlborough, MA, USA).

2.2. Seaweed material and extraction process

The brown seaweed *U. pinnatifida* was hand collected in the central Portuguese coast, on 2018–09-01, at a spring waters low tide, in Praia de Buarcos, Figueira da Foz (40.1766 N, −8.8993 W). In the laboratory the sample was washed to remove all the mucilage. The holdfasts and stipes were removed and discarded. The blades were processed, cut into small pieces and stored in recipients to be lyophilized. After this process, the algae were blitzed until a powder was obtained.

Two decoctions were prepared using 5 g of this powder in 100 mL of distilled water according to the method described by Wang, Jónsdóttir, and Ólafsdóttir (2009). One mixture was boiled for 30 min (W100) and the other was put under agitation at 300 rpm for 24 h at room temperature (W24). The resulting liquid of both extractions was individually centrifuged at 5000*g* for 15 min at 4 °C. After the centrifugation the pellet was discarded, and the supernatants were lyophilized using a Heto PowerDry 3000 lyophilizer. A suspension of the powder *U. pinnatifida,* containing 15 mg/mL in water, was subjected to *in vitro* gastrointestinal digestion using 5 mL of referred suspension and 5 mL of pancreatic juice formed as described in Porfírio et al. (2010). The mixture was stirred, incubated in a 37 °C bath for 24 h and centrifuged at 5000*g*, for 5 min and the supernatant was lyophilized (Wdig). Throughout the study all the extracts were stored at −20 °C before all the tests.

2.3. Mucilages' precipitation

The precipitation of mucilages with ethanol was performed on the two extracts of *U. pinnatifida* previously obtained, according to the methodology described by Guedes et al. (2019).

2.4. Solid phase extraction (SPE)

The purification of the W100 extract using solid-phase extraction as described in André et al. (2020) resulted in a new extract rich in phlorotannins (W100-SPE).

2.5. Compound identification by liquid chromatography-high resolution tandem mass spectrometry (LC-HRMS/MS)

The analysis of *U. pinnatifida* extracts was performed by LC-HRMS/MS in an Elute OLE UHPLC system coupled to a quadrupole time-of-flight (QqToF) Impact II mass spectrometer equipped with an electrospray ionization (ESI) source (Bruker Daltonik GmbH, Bremen, Germany). 5 μL of a 5 mg/mL solution of W100 and W100-SPE extracts and 5 μL of a 1 mg/mL solution of Wdig extract were injected.

Chromatography separation was performed on an Intensity Solo 2 column (1.8 μm C18 100 × 2.1 mm) (Bruker Daltonik GmbH), at a flow rate of 250 μL/min and the sampler and the column were maintained at 10 °C and 35 °C, respectively. Mobile phase consisted of 0.1% (v/v) of acid formic in water (A) and 0.1% (v/v) of acid formic in ACN (B) and the elution conditions were as follows: 0 min, 95% A, 5% B; 1.5 min, 95% A, 5% B; 13 min, 25% A, 75% B; 18 min, 0% A, 100% B, 21 min, 0% A, 100% B, 23 min, 95% A, 5% B and 30 min 95% A, 5% B.

The different mass spectra were acquired in ESI positive/negative modes, in full scan mode in the *m/z* 50–1500 range and in data-dependent acquisition mode (auto MS/MS) with a 3 Hz scan. The experimental parameters used were as follows: ion spray voltage, +4.0 kV and −3.5 kV; end plate offset, 500 V; nebulizer gas (N_2), 2 bar; drying gas (N_2), +8.0 e − 4.0 L/min; dry heater, 200 °C. At the beginning of each analysis, internal calibration was executed on the high-precision calibration mode (HPC) using a solution of sodium formate 10 mM. The acquired data were processed by DataAnalysis 4.4 software (Bruker Daltonik GmbH).

2.6. Total phenolic content quantification

Total phenolic compound content was measured spectrophotometrically using gallic acid as a standard (0–500 μg/mL) as described by Henriques et al. (2017) and expressed as mg of gallic acid equivalents per mg of dry extract.

2.7. Antioxidant activity determination

The DPPH scavenging activity of *U. pinnatifida* extracts was evaluated, in triplicate, using the method described by Falé et al. (2013).

2.8. Acetylcholinesterase inhibitory activity

To determine the inhibition of the enzymatic activity of AChE, the method described in Pedro L. Falé et al. (2013) was used.

2.9. HMG-CoA reductase (HMGR) inhibitory activity

HMGR inhibitory activity of *U. pinnatifida* extracts was determined based on spectrophotometric measurements (at 340 nm), that represent the oxidation of NADPH, following the HMG-CoA Reductase Assay Kit protocol provided by Sigma.

2.10. Cytotoxicity studies in Hep-G2 cell line

Hep-G2 cells (ATCC#HB-8065), from human hepatocellular carcinoma, were cultured at 37 °C using a CO_2 incubator in RPMI medium supplemented with 10% FBS, 1% pen-strep and 1% L-glutamine. The cells were grown in T75 flasks (Corning®) and the medium was changed every 48/72 h. The cytotoxicity of W100 and Wdig was assessed using the MTT viability test described by Mosmann (1983). Briefly, a cell suspension with a final concentration of 50×10^4 cells/mL was prepared in medium without FBS, from which 100 μL were seeded in 96-well plates and incubated for 48 h in the oven. After this time, the culture medium was removed and 100 μL of the extracts W100 and Wdig dissolved in RPMI medium were applied to each well at different concentrations (0.1–2.0 mg/mL) for 24 h. The culture medium was removed before applying 100 μL of the MTT solution in RPMI medium (0.5 mg/mL).

2.11. One-dimension polyacrylamide gel electrophoresis (SDS-PAGE)

In order to study the protein profile, Hep-G2 cells were cultured in T75 flasks. One of the flasks was used as a control (RPMI medium without FBS) and to the others the samples W100 (0.5 mg/mL) and Wdig (0.2 mg/mL), dissolved in the same medium, were added. Two simultaneous studies were carried out: in one the T75 flasks were left to incubate for 4 h in the oven and in the other for 24 h. After this time, cells were scraped, centrifuged for 5 min at 10,000*g*, the supernatant was discarded and 500 μL of lysis buffer (5.05 mL H_2O; 1.15 mL 0.5 M Tris-HCl buffer; 61 mg DTT, pH = 6.8) were added. Cells were then lysed by sonication for 10 min, centrifuged for 5 min at 10,000*g* and the supernatant from the different samples was collected. Proteins of the supernatants were quantified following the “2-D Quant kit” procedure provided by GE Healthcare. Electrophoresis separation was carried out at 100 V for 1 h45, using a 4–10% acrylamide-bis gel, where 5 μL of molecular weight marker and 20 μL of each sample (containing approximately 20 μg protein) were applied. The gel was stained with a solution of Coomassie R-250 40%, methanol 40% and glacial acetic acid 10% for 1 h. After bleaching (solution of 10% glacial acetic acid, 40% methanol and 50% distilled water) the gel was photographically recorded using the ImageQuantTL8.1. (Cytiva, Uppsala, Sweden) device and the gel bands were treated using ImageJ software.

2.12. Statistical analysis

Throughout the experimental work, the results are expressed as average ± standard deviation. Statistical differences between groups were evaluated by one-way analysis of variance (ANOVA), available in Microsoft Office 2013® software. In the statistical treatment, a probability level of $p \leq 0.05$ was used as a significant difference.

3. Results

Several extracts of *U. pinnatifida* were performed using three different methods: boiled in water after being washed and trimmed (W100), similar to a soup preparation, extracted with water at room temperature (W24) and dry and treated with digestive enzymes as eaten raw in a salad (Wdig). In order to remove the mucilages that can be co-extracted with water (Wollgast & Anklam, 2000), a portion of both aqueous extracts was subjected to ethanol precipitation before being dried (W100 w/o mucilages and W24 w/o mucilages). Additionally, to promote the purification of phlorotannins, a sample of W100 extract was subjected to a solid phase extraction (SPE) obtaining the W100-SPE extract.

3.1. Total phenolic content of several extraction processes

The phenolic content of the different *U. pinnatifida* extraction processes, boiling water (W100), room temperature (W24) and dry alga digestion (Wdig), was determined and the results are shown in Table 1. The mucilages withdrawal process (W100 w/o mucilages and W24 w/o mucilages) resulted in an increase of 44% and 58% on the phenolic content at 100 °C, and 24 °C, respectively. The dry extract treated with digestive enzymes originated an extract with higher content of phenolic compounds relatively to the other *U. pinnatifida* extracts (Table 1). The withdrawal of insoluble ethanol compounds like polysaccharides can leave behind small hydrophilic alga metabolites, like sugars or amino acids. W100 extract was also purified using solid revers-phase extraction (SPE) where hydrophilic compounds can also be discarded. W100-SPE showed an 8-fold increase in the phenolic content of the extract. Non-phenolic material was increasing the global mass of the extract and with its withdrawal we may see an improvement of the bioactivity in future tests.

3.2. Capacity to capture free radicals (antioxidant activity)

The antioxidant activity was measured using DPPH method and the results are presented in Table 1. All the extracts were used at an equal

Table 1
Total phenols and bioactivities of several aqueous extractions of *U. pinatifida*. The antioxidant activity was determined with 0.1 mg/mL and 1 mg of extract/mL of each process for the enzyme inhibitory activities.

Extracts	Total Phenols (µg GAE/mg extract)	Antioxidant activity (0.1 mg/mL) (%)	AChE (1 mg/mL) (%)	HMGR (1 mg/mL) (%)
W100	2.36 ± 0.12^{a}	18.79 ± 0.45^{a}	23.59 ± 1.18^{a}	21.23 ± 1.18^{a}
W100 w/o mucilages	3.41 ± 0.16^{b}	14.59 ± 0.49^{b}	14.30 ± 1.85^{b}	4.68 ± 0.98^{b}
W24	3.02 ± 0.16^{b}	15.23 ± 0.73^{b}	12.76 ± 0.55^{b}	15.09 ± 1.03^{c}
W24 w/o mucilages	4.80 ± 0.11^{c}	14.27 ± 0.23^{b}	18.73 ± 0.86^{c}	7.97 ± 0.83^{d}
Wdig	17.89 ± 0.44^{d}	9.79 ± 0.74^{c}	16.04 ± 0.87^{b}	6.44 ± 0.91^{d}
W100-SPE	19.16 ± 0.53^{e}	27.44 ± 0.10^{d}	52.44 ± 0.7^{d}	50.63 ± 0.87^{e}

(a–e) superscript letters correspond to statistic differences at confidence level of 95% ($\alpha = 0.05$).

concentration, 0.1 mg/mL in order to have a first insight into their antioxidant capacity. The extract with higher antioxidant activity was the one obtained with boiling water (W100). In the water extract at room temperature (W24), the antioxidant activity was 18% lower. The enzymatic digestion of *U. pinnatifida* originated an extract with a lower activity.

With SPE purification the antioxidant activity increased 46% relatively to W100, meaning that there were several compounds present in the extract without antioxidant activity that contribute to the weight of the extract and once discarded the activity increased.

3.3. *Acetylcholinesterase inhibitory activity*

The AChE inhibitory activity of the extracts was determined, and the results are indicated in Table 1. The compounds present in W100 extract had the highest inhibitory activity, approximately 24%, at a concentration of 1 mg/mL, therefore this is a promising extract whose purification can give a better result. In fact, after SPE purification 1 mg/mL caused a reduction in the enzyme activity of 50%. W24 gave an inhibition of approximately 13% at the same concentration. The digestive enzyme pancreatin originated an extract with low enzyme inhibitory activity, 16%, meaning that most of the compounds obtained with the enzyme digestion are not good AChE inhibitors. The AChE inhibitory capacity of phloroglucinol, the starting unit of phlorotannins biosynthesis was evaluated by using a standard compound. Using also 1 mg/mL a 3% enzyme reduction in activity was determined, meaning that it has a very low inhibitory capacity for AChE.

3.4. *HMGR inhibitory activity*

The inhibition of the enzyme HMGR is used to treat people having hypercholesterolemia. The results presented in Table 1 indicate that the extract obtained with boiling water (W100) had higher activity than the one obtained at room temperature (W24), and that the withdrawal of mucilage reduced the enzyme inhibitory capacity. Using 1 mg/mL of W100-SPE, the inhibitory activity showed value of 50% attaining an increase of 138% relatively to the non-purified extract. This means that in W100 there are compounds with HMGR inhibitory activity.

3.5. *Cell proteome effect (preliminary tests)*

Since the liver is the main organ for cholesterol biosynthesis, liver cells were used to study the effect of *U. pinnatifida* extract on changes in the cellular proteome. Hep-G2, a human liver cell line, was chosen as they are widely used in studies of cytotoxicity and drug metabolism (Collins et al., 2019). The effect of W100 and Wdig extracts were studied during 4 h and 24 h in an attempt to verify the effect of prolonged consumption. Before studying the effect on cell proteome, the extracts' cytotoxicity was first analyzed. In this assay *U. pinnatifida* extracts were used in concentration ranging from 0.1 to 2.0 mg/mL in contact with Hep-G2 cells and an IC_{50} of 2.0 mg/mL and 0.9 mg/mL for W100 and Wdig extracts were obtained, respectively.

The values obtained in the present study indicated that there is no cytotoxicity towards this type of cells. There are no registers that *U. pinnatifida* could be cytotoxic to any type of cells. So the Hep-G2 cells were put into contact with 0.5 mg/mL for each extract during 4 h and 24 h and compared with control cells during the same period to analyze any possible proteome changes. SDS-PAGE can be seen in Fig. 1a.

Several changes can be seen even on control cell proteins after 24 h relatively to the cells staying 4 h in the culture medium. The effect of W100 and Wdig was quantified by determining the band intensities using ImageJ program, which also allows the calculation of the relative changes in intensities (Fig. 1b left and right side, respectively).

There are proteins with 148, 48, 37 and 21 kDa that have several changes not only with the extract but also after staying for 24 h in the culture medium, disappearing, while proteins with molecular weight 156, 55 and 30 kDa appear after 24 h. Analyzing the effect after 4 h digestion, equivalent to the digestive process, it can be seen that W100 extract produced a decrease in protein intensity with molecular weight 148, 48 and 21 kDa. The digested extract (Wdig) produces a decrease in proteins with 65, 48 and 21 kDa.

3.6. *Tentative identification of the compounds by LC-HRMS/MS*

In order to explain the differences in the biological activities, the identification of the chemical components of each extract was carried out by LC-HRMS/MS. W100, Wdig and W100-SPE extracts were analyzed in both negative and positive modes, Fig. 2a and b, respectively.

The chromatograms indicated fewer compounds in the negative mode than in the positive one for the three extraction conditions and W100 was the extract with the least number of compounds, which is in accordance with the total phenolic content. All the identifications that were carried out had into consideration the compounds present in the water which was used to dissolve the samples. Therefore, the peaks corresponding to these compounds were discarded in the compound identification of the samples. Only compounds with intensity higher than 3000 were identified. Compounds present in W100, Wdig and W100-SPE extracts are indicated in Tables 2–4 respectively. Thirteen compounds in negative mode and forty-eight in positive mode were identified for W100 extract, five in negative mode and thirty-seven in positive mode for Wdig and thirteen in negative mode and one hundred and twenty in positive mode for W100-SPE. In all the samples the compounds were divided into phlorotannins (PL), small peptides (Pp) and flavonoids and carotenoids were grouped as secondary metabolites (S). The compounds were grouped according to their number per group, Fig. 3.

W100 extract showed all type of compounds, whereas Wdig mainly small peptides, one phloroglucinol derivative and other secondary metabolites. The W100-SPE extract showed higher number of phloroglucinol derivatives. The digestion increased substantially the number of small peptides and secondary metabolites, like flavonoid and cinnamic acids derivatives.

The phlorotannins found in the *U. pinnatifida* extract oscillated between 208 and 658 Da. Most of them showed a linear structure of fuhalol type. The peptides had three or four amino acid residues with molecular weight ranging from 314 to 587 Da. These peptides had aromatic amino acids in their structure (Phe, Tyr and Trp). The compounds with higher intensities in the extracts were primary metabolites, not shown in these Tables (around 2.7×10^5; 5.6×10^5, 8.8×10^5 in W100, Wdig and

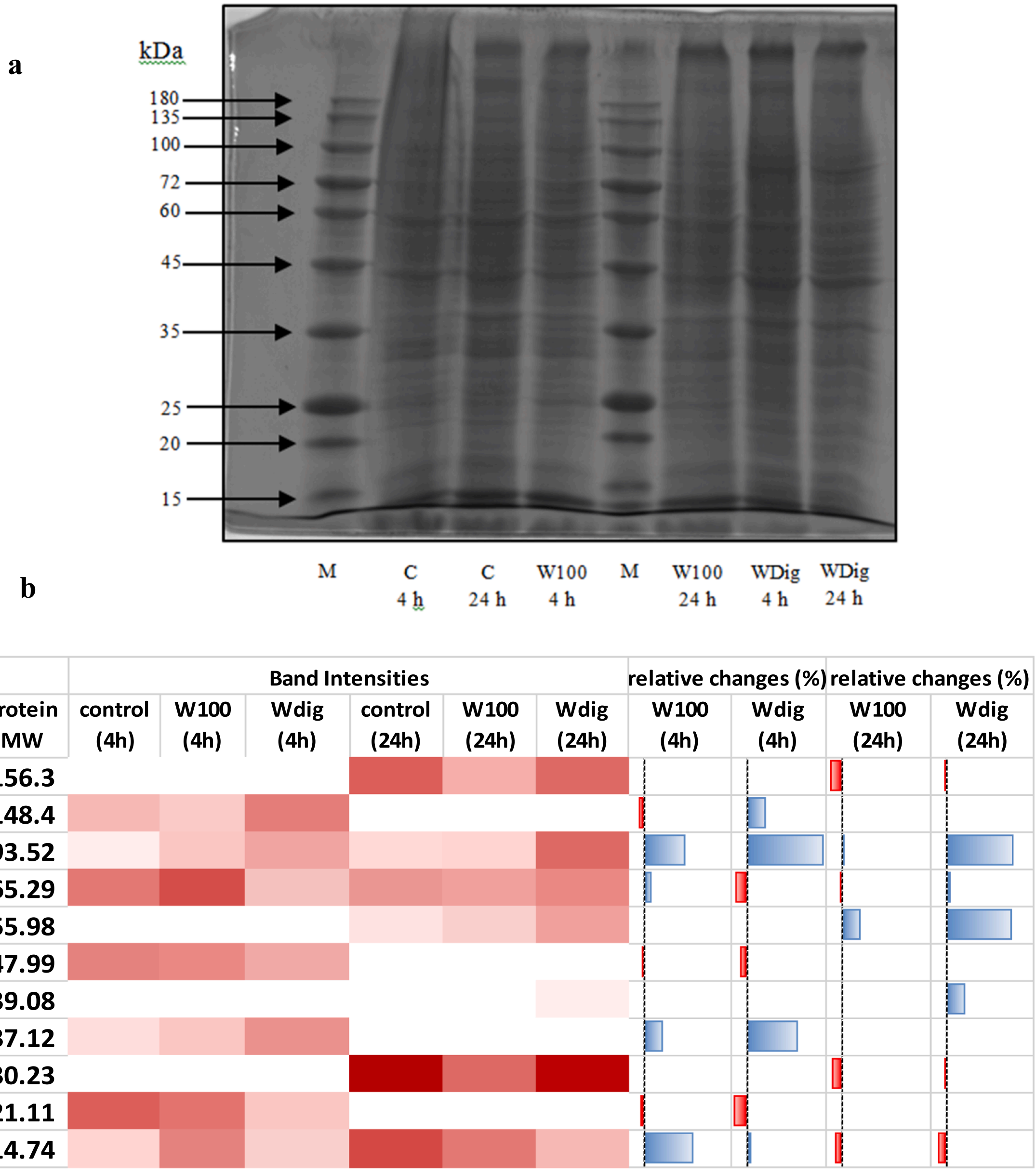

Fig. 1. SDS-PAGE of Hep-G2 proteome under the effect of W100 and Wdig extracts for 4 h and 24 h. In all the lanes 20 μg of protein was applied. (a) gel electrophoresis image; (b) Quantification of protein bands Left side: areas of the band of each protein with molecular weight calculated according to the protein markers; right side: changes relatively to the control at 4 h and 24 h growth. (blue- increase in area; red-decrease in area).

W100-SPE, respectively). The intensity of phlorotannins derivatives in W100 extract oscillated between 1.5 × 10^4 and 1.6 × 10^5. In the Wdig extract the phloroglucinol had an intensity of 2.7 × 10^4 and after purification, W100-SEP phloroglucinols had an intensity of 1.1 × 10^4 and 6.5 × 10^5.

4. Discussion

The bioactivities analyzed with *U. pinnatifida* extracts indicated a high activity of the SPE purified extract, which correlated with the highest value of total phenolic compounds. The amount of phenolic compounds found in these extracts was lower comparatively to those reported in the bibliography for the same alga, 10.7 mg/mg dry weight (Dong et al., 2019), but the extraction conditions carried out in the present work are mild conditions since only water was used. However, comparing with studies where brown algae extracts were also obtained under mild extraction processes, the amount of phenolic compounds is identical (André et al., 2020). Mucilages can be co-extracted with water (Wollgast & Anklam, 2000) and can be withdraw by precipitation with ethanol (Edmond Ghanem et al., 2010). This precipitation resulted in an increase of the total phenol content. Nevertheless, the highest increase in total phenol content was observed with the SPE purification, meaning that several hydrophilic compounds were in fact withdrawn from the extract (Lopes et al., 2018), through the use of this technique.

In order to explain the differences in the bioactivities with the chemical composition, the three extracts were analyzed by LC-HRMS/MS. In fact, more compounds appeared in the chromatogram of W100-SPE and this extract showed the highest content in phenolic compounds. The main type of compounds detected in the extracts besides primary metabolites were phlorotannins, small peptides and some flavonoids. Besides the primary metabolites, phlorotannins and peptides were the compounds detected in higher number, mainly in the W100-SPE sample, that is after discarding the very hydrophilic compounds like small sugars and probably some polysaccharides, the more

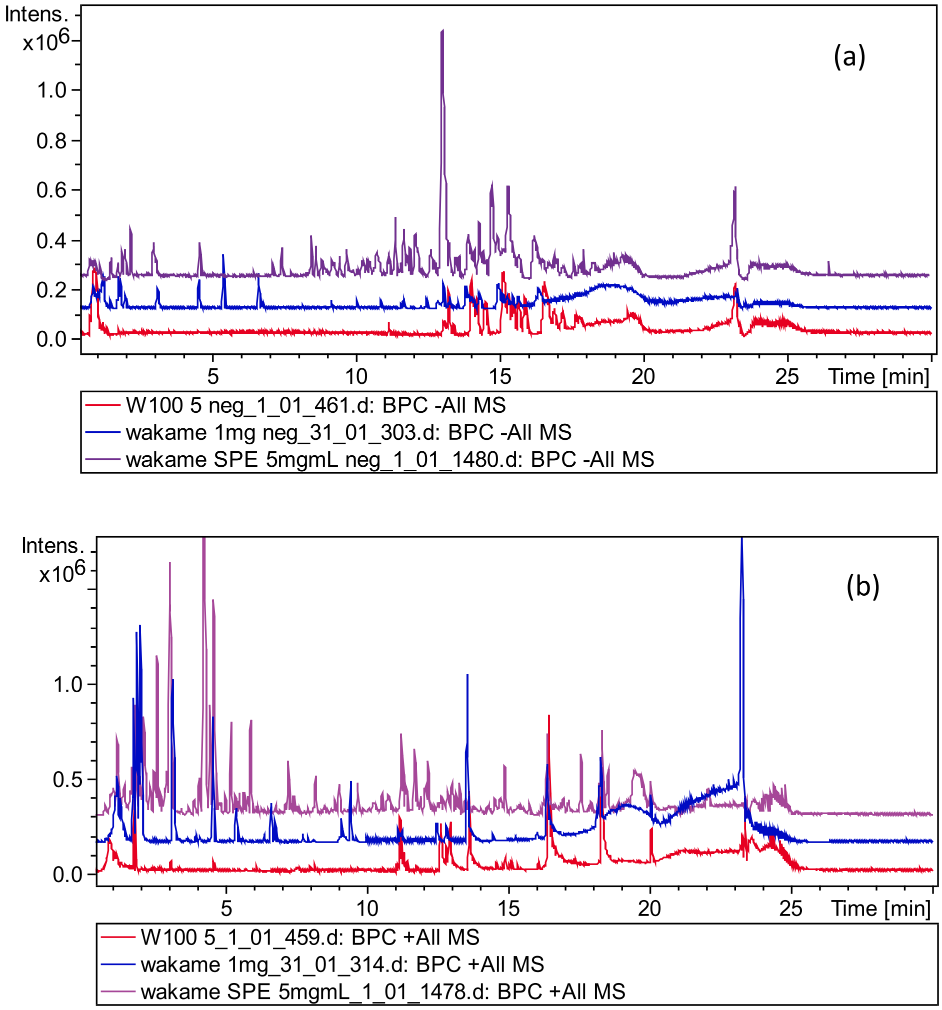

Fig. 2. LC-HRMS/MS chromatograms of *U. pinnatifida* different extracts in negative mode (a) and positive mode (b): boiled alga here W100; digested dry alga (Wdig) here named *U. pinnatifida* and boiled alga purified by SPE (W100-SPE) here named *U. pinnatifida* SPE.

hydrophobic compounds were detected. The digestive process of dry *U. pinnatifida* produces an extract with several primary metabolites, extracting only one phlorotannin. The compounds extracted in higher number, in the digested extract, were small peptides, what is in agreement with the activity of the digestive enzymes contained in the pancreatin mixture (protease, lipase and amylase, indicated by the seller). Pancreatin was not able to extract phlorotannins because it is not able to rupture the cell membrane in order phloroglucinol to come to the medium.

The small peptides were also detected, although in small number in the W100-SPE extract, meaning that they were already present in the soup, some of them appearing in the chromatogram of W100 extract. Comparing the digested alga with the other extracts, the main difference is the existence of only one phlorotannin. Although some activity was noticed in the digested *U. pinnatifida*, what may be ascribed to the presence of small peptides, the low activity detected can be attributed to the non-existence of phlorotannins in a large number, comparatively to the other extracts containing these types of compounds and showing all the bioactivities studied.

Several authors refer the use of enzymes to extract phlorotannins (Habeebullah et al., 2020), but the conditions used are more aggressive, allowing the rupture of the alga cell wall structure. The room temperature used in this study to accomplish the enzyme digestion seems not enough to disrupt most of the cell wall of the algae. It appears that the alga eaten dry in salads may not deliver easily the phlorotannins to the stomach or the intestine, under the mild conditions in the digestive tract. On the other side, the soup (like W100 extract) contains all the ingredients that can be of nutritional value including the small peptides and some phlorotannins, that although not considered as having nutritional value, they may have interesting bioactivities, as for instance those described here.

Phlorotannins have hydroxyl groups bonded to an aromatic ring that can stabilize radicals by resonance structure, showing antioxidant activity. The small peptides detected can also have this capacity (Daliri et al., 2017; Lafarga et al., 2020). Therefore, the DPPH radical scavenging activity was studied. The values obtained using 0.1 mg/ml of extract do not indicate a strong antioxidant activity comparatively to the standards, so no EC_{50} values were determined for the extracts, although the withdrawal of hydrophilic compounds through the SPE purification indicated that several antioxidant compounds are present in the extract, the DPPH scavenging activity increased. These compounds contribute to the mass of the extract without having high antioxidant activity, what leads to a low value for the DPPH test. The withdrawal of mucilage originated a decrease in the antioxidant activity, meaning that there are several compounds participating in this process (Zhong et al., 2019). Comparing the antioxidant activity of *U. pinnatifida* extracts with the activity of phloroglucinol ($EC_{50} = 20.7$ μg/mL) or with the standards butylated hydroxytoluene (BHT), rutin and quercetin with EC_{50} values of 12.6 μg/mL (Henriques et al., 2017), 6.3 μg/mL (Guedes et al., 2019) and 3.2 μg/mL (Guedes et al., 2019) respectively, the alga is not as active as the standards, but comparatively to other brown alga aqueous extracts the activity is of the same magnitude (André et al., 2020) or even

Table 2
Identification proposal of bioactive compounds detected in *U. pinnatifida* boiling extract (W100) by HRMS. Compound number with (+) or (-) indicates that were identified in positive and negative mode.

Rt (min)	Molecular formula	$[M-H]^-$/ $[M+H]^+$	Error (ppm)	Fragments ion (*m/z*); intensity (%)	Tentative identification	Intensity
1.0^-	$C_{12}H_{10}O_4$	217.0481	−6.4	126.90 (9%); 94.92 (100%); 92.92 (33%); 78.95(38%)	Phloroglucinol derivative	17,118
1.3^+	$C_9H_{17}NO_4$	204.1227	1.2	145.04 (8%); 85.03 (100%); 60.08 (17%)	Phloroglucinol derivative	23,667
12.7^+	$C_{22}H_{42}N_6O_4$	455.3341	−0.2	394.11 (1%); 254.16 (1%); 280.19 (2%); 239.16 (100%)	Peptide like structure	66,751
12.9^+	$C_{16}H_{35}NO$	258.2795	−0.6	240.26 (7%); 71.08 (65%); 57.06 (16%)	16-aminohexadecan-1ol	25,082
12.9^+	$C_{20}H_{43}NO_3$	346.3318	−2.5	328.32 (1%); 284.29 (12%); 70.06 (8%); 45.03 (5%)	Phytosphingosine	13,253
12.9^+	$C_{26}H_{42}O$	371.3271	2.8	268.26 (100%); 123.11 (1%); 97.10 (15%); 95.08 (2%)	Nor-cholestediene-ol	91,046
13.1^-	$C_{19}H_{22}O_3$	297.1521	−8.1	183.01 (69%); 119.05 (28%); 79.95 (33%)	Phloroglucinol derivative	32,548
13.3^-	$C_{15}H_{22}O_4$	265.1475	−12.9	124.80 (1%); 96.95 (100%); 79.95 (17%)	Phloroglucinol derivative	62,236
14.1^-	$C_{20}H_{24}O_3$	311.1679	−10.1	183.01 (69%); 119.05 (28%); 79.95 (33%)	Phloroglucinol derivative	146,833
14.5^-	$C_{21}H_{26}O_3$	325.1826	−7.2	212.05 (2%); 197.02 (32%); 183.01 (25%); 125.03 (1%); 133.06 (23%); 125.03 (1%)	Phloroglucinol derivative	135,073
14.5^-	$C_{17}H_{26}O_5$	309.1730	−9.0	216.00 (3%); 183.01 (18%); 96.95 (100%); 79.95 (40%)	Hymenolide	52,359
14.7^+	$C_{11}H_{18}N_6O_2$	267.1565	−4.4	236.71 (6%); 196.05 (10%); 136.05 (3%); 117.09 (13%); 85.06 (8%); 57.06 (27%)	Purine derivative	16,751
14.7^+	$C_{17}H_{37}N$	256.3003	−1.1	209.16 (1%); 199.15 (1%); 60.08 (14%); 45.05(4%)	Isoheptadecylamine	21,363
14.9^+	$C_{23}H_{47}NO_4$	402.3576	−1.6	283.23 (98%); 57.06 (21%); 71.08 (9%)	Hydroxyethylamino-methyloctadecanoate	11,007
14.9^+	$C_{18}H_{37}NO_3$	316.2845	−3.8	255.23 (22%); 212.16 (7%); 106.08 (100%); 88.05 (75%); 70.06 (44%); 57.06 (21%)	Hydroxyethyltetra-decamide	14,579
15.2^-	$C_{19}H_{30}O_6$	353.1991	−5.0	183.14 (1%); 138.97 (1%); 123.97 (3%); 98.96 (4%); 96.96 (56%)	Phloroglucinol derivative	17,018
15.2^+	$C_{12}H_{20}N_6O_2$	281.1729	−1.5	149.02 (10%); 95.08 (7%); 69.06 (7%); 57.06(16%)	Purine derivative	228,407
15.7^-	$C_{17}H_{26}O_4$	293.1776	−6.2	220.14 (1%); 183.01 (1%); 165.02 (1%); 96.95 (100%)	Phloroglucinol derivative	17,062
16.1^+	$C_{14}H_{24}N_6O_2$	309.2035	−0.5	111.11 (9%); 69.07 (12%)	Phloroglucinol derivative	274,672
16.5^+	$C_{16}H_{27}NO_4$	298.207	−19.2	239.14 (2%); 235.1 (13%); 181.07 (3%); 111.11 (14%); 69.06 (100%)	Phloroglucinol derivative	19,516
16.5^+	$C_{14}H_{26}N_4O_4$	315.2004	7.2	269.05 (4%); 212.16 (6%); 111.11 (20%); 69.06 (100%)	Lys-Ala-Pro	10,402
16.5^+	$C_{16}H_{24}N_2O_5$	325.1778	−6.8	111.11 (13.5%); 69.06 (100%); 55.07 (22%)	Phloroglucinol derivative	67,297
16.5^+	$C_{30}H_{40}O_3$	449.3032	4.1	271.11 (23%); 227.14 (1%); 181.03 (14%); 163.02 (14%); 111.11 (51%); 69.06 (100%); 55.05 (14%)	Fucoxanthin derivative	58,240
16.6^-	$C_{22}H_{28}O_3$	339.1991	−3.6	183.14 (44%); 119.05 (41%); 79.95 (29%)	Phloroglucinol derivative	133,623
20.1^+	$C_{24}H_{38}O_4$	391.2844	−0.3	167.03 (18%); 149.02 (100%); 71.08 (28%); 57.08 (34%)	Phloroglucinol derivative	130,520
20.1^+	$C_{26}H_{36}O_4$	413.2662	7.5	301.14 (18%); 189.01 (4%)	Phloroglucinol derivative	60,746

Table 3
Identification proposal of bioactive compounds detected in *U. pinnatifida* digested extract (Wdig) by HRMS. Compound number with (+) or (-) indicates that were identified in positive and negative mode, respectively.

Rt (min)	Molecular formula	$[M-H]^-$/ $[M+H]^+$	Error (ppm)	Fragments ion (*m/z*); intensity(%)	Tentative identification	Intensity
1.0^-	$C_{18}H_{18}O_9$	377.0846	5.4	198.91 (16%); 179.05 (10%); 97.02 (10%); 137 (10%)	Flavonoid derivative	39,900
1.9^+	$C_9H_8O_3$	165.0550	−2.3	123.04 (84%); 147.04 (7%)	4-Hydroxycinnamic acids	191,011
2.0^+	$C_{22}H_{26}N_2O_7$	431.1765	5.4	116.07 (55%); 316.01 (61%); 196.06 (23%)	TyrMe-Ile-OH	9640
2.1^+	$C_{12}H_{21}N_3O_6$	304.1486	5.5	72 0.08 (100%); 187.10 (10%); 159.07 (2%)	GluGlyVal	6961
4.1^+	$C_{10}H_{18}N_2O_5$	247.1285	1.4	132.10 (70%); 201.12 (44%); 116.07 (28%); 88.03 (83%)	AspLeu	8338
4.1^+	$C_{14}H_{18}N_2O_4$	279.1337	−4.2	234.11 (2%); 136.0 (100%); 116.07 (63%); 91.05 (66%)	ProTyr	35,520
4.2^+	$C_{11}H_{22}N_2O_3$	231.1702	0.0	86.09 (100%); 130 (2%); 57.05 (3%)	ILeVal	13,626
4.9^+	$C_{11}H_{20}N_2O_3$	229.1548	2.4	116 (74%); 86 (100%)	ProLeu	28,762
4.7^+	$C_{12}H_{24}N_4O_4$	409.1866	1.1	188.07 (100%); 159.09 (12%); 132.08 (7%)	GlyTrpPhe	11,870
5.5^+	$C_{15}H_{17}N_3O_5$	320.1238	9.5	188 (100%); 130 (55%); 146 (48%)	AspTrp	10,390
5.5^-	$C_{18}H_{10}O_8$	353.0279	6.8	165.01 (54%); 121.02 (100%)	Phloroglucinol derivative	8331
5.7^+	$C_{21}H_{36}N_4O_5$	425.2760	−3.0	312.19 (52%); 211.14 (46%); 183.14 (16%)	IleProValPro	22,534
5.7^+	$C_{14}H_{18}N_2O_3$	263.1382	−7.2	120.08 (100%); 103.05 (37%); 91 (14%); 116.07 (66%)	ProPhe	9876
6.2^+	$C_{19}H_{24}N_4O_5$	389.1819	−0.1	246.12 (100%); 159.09 (36%); 274.11 (57%); 357.87 (4%)	TrpSerPro	7208
6.7^+	$C_{16}H_{17}N_5O_7$	392.1146	12.9	268.07 (100%); 235.05 (2%); 197.01 (6.2%); 224.04 (6%)	His-Asn-OH	19,685
6.8^+	$C_{25}H_{26}O_{13}$	535.1344	25	179.00 (47%); 517.12 (45%); 447.07 (30%); 411.09 (22%)	Dicaffeoylquinic derivative	17728/
9.5^+	$C_{17}H_{14}O_6$	315.0833	9.3	146.01 (22%); 245.06 (96%); 174.05 (20%)	Phloroglucinol derivative	27,120
12.6^+	$C_{14}H_{22}O_3$	239.1618	10.1	163.07 (15%); 181.04 (8%); 57 (18%); 123.06 (7%); 136 (10%); 111.04 (13%)	Laevinol	114,718
12.6^+	$C_{23}H_{42}N_4O_5$	455.3343	25	239.16 (100%)	ProIleIleIle	37,898
14.5^+	$C_{20}H_{36}N_6O_6$	457.2769	−1.4	412.93 (1%); 327.34 (1%); 111.11 (1%)	ProValAsnLys	19,233

higher than that obtained with some green algae (Farasat et al., 2014) that are reported to have antioxidant activity.

On what concerns AChE inhibition activity, it can be seen that W100-SPE showed 50% inhibition when using 1 mg/mL. Comparing the activity of *U. pinnatifida* with the activity of another brown alga *Fucus vesiculosus*, from the Tagus river estuary, and prepared at 100 °C, after SPE purification, a similar inhibitory activity was obtained, that is 60% when using 1 mg/mL (André et al., 2020). The phlorotannins (Kannan et al., 2013) and the small peptides (Prasasty, Radifar, & Istyastono, 2018) have also been described as able to inhibit this enzyme. Compounds having a benzene ring can fit inside the enzyme active site with a low binding energy (Guedes et al., 2019) due to π- π interactions that may be established between the Phe residue at the enzyme active site entrance, that is the Peripheral Anionic Site (PAS) (Branduardi,

Table 4

Identification proposal of bioactive compounds detected in SPE purified *U. pinnatifida* extract (W100-SPE) by HRMS. Compound number with (+) or (−) indicates that were identified in positive and negative mode, respectively.

Rt (min)	Molecular formula	$[M-H]^-$/ $[M+H]^+$	Error (ppm)	Fragments ion (*m/z*); intensity (%)	Tentative identification	intensity
2.1^{+}	$C_{18}H_{28}N_6O_8$	457.2041	2.3	894.04 (100%); 440.07 (11%); 240.09 (48%); 218.11 (39%); 212.10 (8%); 129.06 (32%)	HisThrAlaGlu	10,508
2.6^{+}	$C_{19}H_{18}O_4$	311.1274	1.2	202.10 (8%); 181.06 (100%); 110.03 (21%)	Moracin C	43,775
3.2^{+}	C_8H_9N	120.0809	−2.0	103.05 (100%); 91.05 (28%); 62.03 (11%)	Vinylaniline	826,133
3.2^{+}	$C_{14}H_{19}N_2O_7S$	359.0911	−1.2	342.06 (16%); 313.08(3%); 296.05 (23%); 208.04 (13%); 195.01 (15%); 164.99 (14%)	Miraxanthin	13,145
5.4^{+}	$C_{18}H_{26}N_4O_6$	395.1917	2.1	215.12 (100%); 187.14 (12%); 141.09 (51%); 95.08 (79%)	GlyValGlyTyr	12,487
5.8^{+}	$C_{19}H_{34}N_4O_6$	415.2542	5.0	370.21 (1%); 239.14 (1%); 89.05 (39%); 45.03 (100%)	ProSerValLeu	15,665
5.8^{+}	$C_{19}H_{37}N_5O_6$	432.2803	2.6	415.25 (59%); 327.20 (5%); 221.13 (10%); 89.05 (41%)	LysThrLeuAla	17,504
5.8^{+}	$C_{21}H_{32}N_4O_6$	437.2361	−7.5	392.23(1%); 131.07 (1%); 115.01 (1%); 86.09 (2%)	LeuGlythrPhe	26,052
7.3^{+}	$C_{22}H_{35}N_5O_5$	450.2754	−9.6	406.30 (1%); 334.49 (1%); 320.49 (1%); 177.11 (16%); 155.09 (10%); 133. (32%); 129.1 (1%)	LysTrpLeu	41,919
8.0^{+}	$C_{20}H_{34}N_{10}O_{10}$	575.2639	−0.5	557.25 (15%); 517.16 (1%); 443.1 (1%); 428.21 (1%); 229.12 (2%)	AspArgGLy peptidic derivative	13,245
8.3^{+}	$C_9H_{14}O_3$	171.1018	−1.3	153.09 (9%); 111.04 (100%); 83.04 (15%)	Ethyl-methoxy hydrofluroglucinol	36,851
8.2^{+}	$C_{34}H_{40}O_8$	577.2752	1.2	542.25 (1%); 532.26 (21%); 517.20 (2%); 430.22 (1%); 347.22 (1%); 291.14 (100%); 251.06 (71%); 81.06 (14%)	Phloroglucinol derivative	15,875
8.7^{-}	$C_{14}H_{20}O_5$	267.1197	−0.3	223.13 (3%); 163.5 (23%); 96.95 (19%)	Phloroglucinol derivative	90,176
8.9^{-}	$C_{11}H_{14}O_4$	209.0818	−1.1	141.08 (1%); 96.95 (100%); 79.95 (83%)	Phloroglucinol derivative	11,491
8.9^{-}	$C_{18}H_{28}O_5$	323.1812	16.1	233.15 (89%); 197.11 (4%); 125.04 (2%); 111.07 (8%); 83.07 (10%); 73.02 (15%)	Phloroglucinol derivative	11,228
8.9^{-}	$C_{18}H_{32}O_5$	327.2112	16.4	233.15 (100%); 183.13 (8%); 73.02 (25%)	Phloroglucinol derivative	24,645
10.0^{+}	$C_{24}H_{38}N_8O_8$	567.2780	5	385.01 (2%); 439.19 (2%); 232.14 (1%); 185.04 (4%); 157.04 (1%)	ArgThrGlnTyr	16,345
10.0^{+}	$C_{28}H_{38}N_4O_6$	527.2852	−2.2	509.27 (16%); 492.27 (2%); 408.24 (2%); 365.23 (88%); 273.10 (45%)	ValPheValTyr	15,414
10.3^{+}	$C_{18}H_{28}O_4$	309.2060	2.5	291.19 (12%); 273.18 (22%); 125.09 (5%); 157.10 (12%); 129.06 (13%); 81.06 (96%); 91.05 (61%)	Dodecanoylphloroglucinol	33,272
10.6^{+}	$C_{13}H_{18}O_3$	223.1331	6.1	206.08 (11%); 193.09 (14%); 121.10 (12%); 83.08 (100%)	Phloroglucinol derivative	24,657
10.6^{+}	$C_{15}H_{22}O_5$	283.1538	0.7	223.13 (76%); 181.12 (67%); 125.09 (100%); 85.02 (40%)	Phloroglucinol derivative	72,139
10.6^{+}	$C_{28}H_{38}N_6O_8$	587.2837	−2.2	346.02 (5%); 305.13 (100%)	TyrLysAsnTyr	10,182
11.0^{+}	$C_{18}H_{30}O_4$	311.2224	−3.4	293.20 (16%); 275.19 (21%); 187.11 (3%); 141.16 (5%); 125.09 (4%); 81.09 (100%); 61.05 (42%)	Phloroglucinol derivative	56,347
11.0^{+}	$C_{20}H_{30}O_5$	351.2149	6.3	333.20 (45%); 315.19 (1%); 173.13 (1%); 157.10 (1%); 71.04 (2%)	Phloroglucinol derivative	70,457
11.0^{+}	$C_{27}H_{42}O_9$	511.2903	−1.4	348.23 (100%); 257.18 (43%); 173.13 (22%); 81.03 (2%); 73.03 (5%)	Phloroglucinol derivative	12,385
11.3^{+}	$C_{17}H_{26}O_4$	295.1906	−1.5	277.12 (17%); 171.19 (4%); 137.06 (100%); 127.05 (1%); 81.06 (39%)	Phloroglucinol derivative	13,603
11.3^{+}	$C_{18}H_{39}NO_3$	318.3006	−0.5	300.29 (1%); 283.29 (1%); 266.28 (1%); 256.26 (18%)	Phloroglucinol derivative	44,256
11.3^{+}	$C_{20}H_{26}O_2$	331.1875	8.0	277.19 (20%); 273.18 (12%); 259.17 (2%); 231.17 (5%); 81.07 (9%)	Phloroglucinol derivative	69,454
11.4^{+}	$C_{20}H_{32}O_5$	353.2303	5.0	335.21 (47%); 317.21 (8%); 199.11 (3%); 159.11 (2%); 143.08 (3%); 81.06 (9%)	Phloroglucinol derivative	12,255
11.5^{+}	$C_{22}H_{30}O_5$	375.2147	4.4	357.20 (15%); 197.09 (2%); 170.91 (2%); 142.07 (2%); 127.91 (2%); 72.08 (2%)	Phloroglucinol derivative	10,736
11.8^{+}	$C_{15}H_{20}O_2$	233.1539	0.0	215.14 (6%); 145.10 (8%); 131.08 (56%); 105.07 (22%); 91.05 (100%); 69.06 (30%)	Phloroglucinol derivative	11,205
11.8^{+}	$C_{20}H_{28}O_4$	333.2434	7.0	315.19 (5%); 209.13 (1%); 91.10 (2%); 91.05 (2%); 81.07 (1%); 69.06 (2%)	Phloroglucinol derivative	73,700
11.8^{+}	$C_{23}H_{24}O_3$	349.1766	9.2	331.16 (20%); 312.22 (13%); 294.21 (24%); 121.06 (12%); 91.05 (27%)	Phloroglucinol derivative	12,742
12.0^{+}	$C_{20}H_{30}O_4$	335.2188	8.0	317.20 (1%); 301.21 (1%); 253.19 (1%); 221.13 (1%); 163.07 (1%)	Phloroglucinol derivative	73,700
12.0^{+}	$C_{18}H_{32}O_4$	313.2370	1.1	277.21 (46%); 259.20 (15%); 237.10 (32%); 209.11(27%); 111.08 (10%); 81.06 (79%); 57.07 (37%)	Phloroglucinol derivative	40,679
12.2^{+}	$C_{45}H_{50}O_3$	639.3844	−1.8	623.38 (1%); 333.20 (21%); 331.18 (100%); 173.09 (1%); 149.09 (1%)	Phloroglucinol derivative	12,844
12.7^{+}	$C_{22}H_{28}O_4$	357.2030	8.3	339.19 (1%); 237.14 (1%); 206.12 (2%); 81.07 (1%)	Phloroglucinol derivative	14,198
13.0^{-}	$C_{15}H_{22}O_4$	265.1439	−0.1	96.95 (100%)	Phloroglucinol derivative	654,716
13.2^{-}	$C_{18}H_{28}O_3$	291.1921	15.3	273.18 (22%); 193.12 (86%); 123.08 (20%); 121.09 (31%); 119.05 (19%); 83.04 (22%)	Phloroglucinol derivative	42,195
13.3^{+}	$C_{18}H_{26}O_2$	275.2005	−3.3	257.19 (8%); 239.18 (8%); 203.14 (1%); 147.11 (15%); 133.10 (33%); 91.05 (100%); 67.05(76%); 55.05 (37%)	Phloroglucinol derivative	50,377
13.4^{+}	$C_{27}H_{44}O_9$	513.3058	−7.7	495.29 (8%); 477.28 (2%); 459.27 (3%); 351.25 (100%); 259.20 (78%); 119.12 (25%); 135.11 (13%); 185.11 (11%)	Phloroglucinol derivative	10,338
13.5^{+}	$C_{18}H_{28}O_2$	277.2160	−2.2	259.20 (3%); 241.19 (1%); 109.10 (14%); 107.08 (46%); 97.10 (4%); 83.08 (3%); 81.06 (41%)	Phloroglucinol derivative	68,442
13.5^{+}	$C_{20}H_{28}O_3$	317.2083	14.1	299.20 (45%); 274.71(10%); 243.05 (16%); 139.03 (23%); 132.09 (72%)	Phloroglucinol derivative	22,462
13.6^{+}	$C_{20}H_{28}O_2$	301.2160	1.3	283.20 (12%); 265.19 (7%); 245.15 (18%); 217.12 (7%); 161.13 (8%); 83.08 (8%); 81.06 (62%)	Phloroglucinol derivative	13,155
13.6^{+}	$C_{22}H_{30}O_4$	359.2185	8.2	341.21 (2%); 325.20 (1%); 297.12 (2%); 120.07 (2%); 91.05 (4%)	Phloroglucinol derivative	11,882
14.2^{-}	$C_{20}H_{24}O_3$	311.1638	−4.0	230.02 (3%); 183.00 (49%); 119.04 (37%); 79.95 (40%)	Phloroglucinol derivative	122,135
14.4^{-}	$C_{17}H_{26}O_5$	309.1697	1.0	183.00 (2%); 122.97 (2%); 95.9 (19%); 79.9 (31%)	Phloroglucinol derivative	91,293
14.6^{-}	$C_{21}H_{26}O_3$	325.1791	−2.9	306.9 (1%); 216.00 (3%); 197.02 (16%); 183.00 (22%); 119.04 (15%); 81.95 (2%); 79.95 (21%)	Phloroglucinol derivative	70,635
14.6^{+}	$C_{27}H_{45}N_2O_8P$	557.2983	−0.2	465.25 (4%); 314.27 (24%); 313.27 (100%); 239.23 (45%); 123.11 (6%)	Phosphocholine derivative	27,510
14.8^{+}	$C_{26}H_{50}NO_7P$	520.3394	1.0	258.11 (2%); 184.07 (65%); 124.99 (8%); 104.10 (40%)	Phosphocholine derivative	25,558
14.8^{-}	$C_{19}H_{30}O_6$	353.1951	5.8	182.99 (1%); 96.95 (43%); 79.95 (16%)	Phloroglucinol derivative	58,253
14.8^{-}	$C_{27}H_{40}O_9$	507.2650	−7.1	279.22 (100%); 277.02 (10%); 171.00 (1%); 152.99 (28%)	Phloroglucinol derivative	10,823

(*continued on next page*)

Table 4 (*continued*)

Rt (min)	Molecular formula	$[M-H]^-$/ $[M+H]^+$	Error (ppm)	Fragments ion (*m/z*); intensity (%)	Tentative identification	intensity
15.3^-	$C_{17}H_{28}O_4$	293.1749	2.1	96.95 (100%); 79.95 (17%)	Phloroglucinol derivative	301,592
16.4^+	$C_{18}H_{28}O_4$	309.2035	11.5	222.14 (2%); 111.11 (5%); 97.10 (2%); 69.06 (5%)	Phloroglucinol derivative	124,362
17.6^+	$C_{29}H_{48}O_{15}$	637.3049	2.2	581.24 (24%); 525.18 (14%); 393.08 (37%); 337.02 (52%); 239.04 (6%); 147.11 (19%)	Phloroglucinol derivative	223,251
17.6^+	$C_{29}H_{51}NO_{15}$	654.3320	1.8	637.30 (100%); 581.24 (45%); 525.18 (26%); 469.11 (9%); 393.08 (45%); 337.02 (42%); 239.04 (7%); 149.11 (12%); 57.06 (15%)	Phloroglucinol derivative	115,094
17.6^+	$C_{38}H_{42}O_{10}$	659.2867	−2.0	345.22 (2%); 240.15 (2%); 227.19 (2%); 221.13 (4%); 137.09 (2%); 125.09 (2%)	Phloroglucinol derivative	45,210

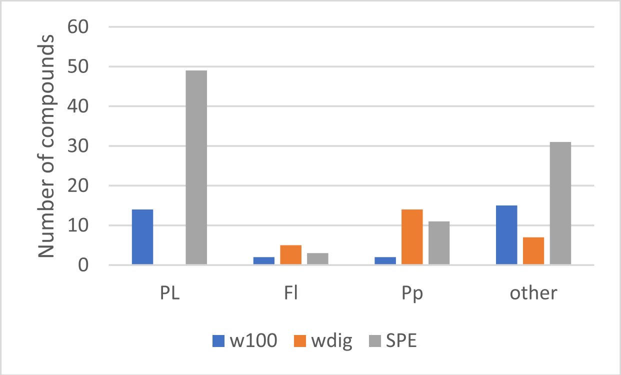

Fig. 3. Total number of phlorotannins (PL), small peptides (Pp) and flavonoids and other secondary metabolites non phlorotannin (S) identified in *U. pinnatifida* extracts (W100, Wdig and W100-SPE).

Gervasio, Cavalli, Recanatini, & Parrinello, 2005). Recently, it was reported that AChE was also inhibited by another brown algae extract also produced as a soup (André et al., 2020). Phloroglucinol itself does not inhibit AChE, what indicates that to inhibit this enzyme a bigger molecular structure is necessary to fit inside the enzyme active gorge. Although no theoretical studies were found with theses phlorotannin molecules, we can speculate that only phlorotannins with two or more phloroglucinols residues may fit inside the enzyme active site, avoiding the entrance of the substrate acetylcholine. On the other side, peptides are also reported to be AChE activity inhibitors (Prasasty et al., 2018). Several small peptides were detected in these extracts that can also contribute to the inhibition effect determined for this enzyme. The IC_{50} was attained when 1 mg/mL of W100-SPE was used.

As algae are reported to have a reduction effect on cholesterol levels (Patil et al., 2018), one possible mechanism to achieve this effect may be to act as inhibitor of cholesterol biosynthesis in a similar manner to statins. HMGR was also inhibited by the *U. pinnatifida* extracts, mainly by W100-SPE, reaching 50% of inhibition (IC_{50}) with 1 mg/mL of extract. Although being a high value for inhibition, it demonstrated that the extract has potential for inhibiting these enzymes once in the digestive tract. The main concern here is the possibility of permeating the intestinal barrier to act on the biosynthesis of cholesterol in the liver. It is known that the intestinal permeation of peptides is restricted to two or three amino acids residues and what concerns phlorotannin there are no studies about the permeability related to the molecular weight or its chemical structures, being linear or branched. Small peptides are usually absorbed with 2–3 amino acids residues (Shen & Matsui, 2019) and in relation to phlorotannins there were no studies found about their intestinal permeability. Nevertheless, during *in vitro* studies, the phlorotannin eckol was able to inhibit HMGR (Yeo et al., 2012).

To study the effect of the extract on cell proteome, the cytotoxicity of the extract had to be first evaluated. The IC_{50} values obtained, 2.0 mg/mL and 0.9 mg/mL for W100 and Wdig, respectively, indicated that they were not cytotoxic (Okonogi et al., 2007) and a value of 0.5 mg/mL of each extract could be used in these tests. The study of the effect of the boiled extract (W100) on the liver cells proteome, simulated by the liver cell line Hep-G2, showed that a modification on cell proteome occurred. Several changes were noticed that deserve future investigation mainly to identify the variations in 148 kDa protein that is precisely the molecular weight of NPC1L1, the protein responsible for the cholesterol transport into the cells (Altmann et al., 2004).

Aqueous extracts of *Fucus vesiculosus*, another brown alga used in food, also containing in its aqueous extracts small peptides and phlorotannins produced a decrease of 40% on cholesterol intestinal permeability in Caco2 cell lines (André et al., 2020). Comparing the effect of 24 h contact with the cells, a simulation of a permanence of the extracts into contact with intestinal cells after an alga meal, a decrease in almost all cell proteins can be noticed. This situation was similar to the one found with plant extracts, also containing phenolic compounds. In this last case the proteins modified were related to cancer, heat-shock proteins, or lipid metabolism (Falé et al., 2012; Guedes et al., 2019; Liu et al., 2013). These changes are being studied at this moment.

5. Conclusions

U. pinnatifida prepared as a soup, boiling with water, transfers to the liquid several bioactive molecules, among which phlorotannins of small molecular weight and small peptides can be referred. In this extract many primary metabolites were also obtained. Phlorotannin had molecular weights between 208 and 658 Da. They were formed by 3–6 phlorogucinol residues and the small peptide by 3–4 amino acid residues giving molecular weight between 314 and 586 Da. To the presence of these compounds, antioxidant activity as well as the capacity to inhibit AChE and HMGR can be recognized. These results mean that *U. pinnatifida* soup can reduce the cholesterol blood level, either by increasing gastrointestinal motility due to the inhibition of AChE or by decreasing its biosynthesis. Alga eaten dry in salads will have more difficulty in liberating its bioactive molecules to the digestive liquid. At least pancreatin was not able to produce a very bioactive extract from dry *U. pinnatifida*. The effect of *U. pinnatifida* extract on liver cells proteome produced several changes in the intensity of some proteins that are being analyzed now. *U. pinnatifida* is a promising alga with several bioactive molecules from which some improvement in human health can be expected.

6. Ethics statement

There were no studies that might be subject to ethic concerns.

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CRediT authorship contribution statement

Bernardo Pedro: Formal analysis, Investigation. **Laura Guedes:** Formal analysis, Investigation, Writing - original draft. **Rebeca André:** Writing - original draft. **Helena Gaspar:** Writing - review & editing. **Pedro Vaz:** Supervision. **Lia Ascensão:** Writing - review & editing. **Ricardo Melo:** . **Maria Luísa Serralheiro:** Conceptualization, Supervision, Supervision, Methodology, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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