J Appl Phycol (2015) 27:519–530
DOI 10.1007/s10811-014-0291-9

Phenolic content and antioxidant activity of fractions obtained from selected Irish macroalgae species (*Laminaria digitata*, *Fucus serratus*, *Gracilaria gracilis* and *Codium fragile*)

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Received: 3 December 2013 / Revised and accepted: 11 March 2014 / Published online: 4 April 2014

Abstract Total phenolic content (TPC) and antioxidant activity of crude and enriched aqueous, ethanol/water and methanol/water extracts from four species of Irish macroalgae *Laminaria digitata*, *Fucus serratus*, *Gracilaria gracilis* and *Codium fragil*e were assessed. The antioxidant activity and TPC of crude and enriched extracts were assessed using the DPPH, FRAP and Folin-Ciocalteu assays. Extracts of *F. serratus* were significantly higher ($p<0.05$) than the other macroalgae. Further enrichment based on liquid-liquid extraction and molecular weight cut-off (MWCO) dialysis generated fractions of low and high molecular weights, (< 3.5 kDa, 3.5–100 kDa, >100 kDa) and hydrophobic with a significant increase in antioxidant activity and TPC ($p<0.05$) compared to the crude extracts. Initial weak activity in the <3.5-kDa fractions for the species *F. serratus* was enhanced following reverse-phase flash chromatography fractionation. Analysis by quadrupole time-of-flight mass spectrometry (Q-Tof-MS) suggests that the <3.5-kDa fractions for *F. serratus* contained a high abundance of low-molecular weight phlorotannins. This study highlights techniques to further enrich fractions with potential for further exploitation, in particular *F. serratus*.

Keywords Macroalgae · Polyphenols · Phlorotannins · Antioxidants · Irish · Mass spectrometry

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Introduction

Ireland has a small but expanding macroalgae harvesting industry dominated largely by brown species of which only a small portion (4 %) is further processed after harvest. In general, the harvested species are used mainly for food or agricultural purposes, and very small quantities are used for secondary processing or in the cosmetics industry. It is likely however that sustainable levels in excess of those currently harvested are available if new markets and uses for them could be found. For example, Hession et al. (1998) estimated that the total sustainable yield of *Ascophyllum nodosum* was 74,845 t per annum; at the time of the report, the actual harvest was 35,850 or 48 % of the potential yield. Werner and Kraan (2004) estimated that there is 3,000,000 t of *L. hypoborea*/*L. digitata* along the coastline of Ireland. Currently, the largest amount of seaweed harvested in Ireland is of *A .nodosum*; however, significant quantities of the species targeted in the present study (*Laminaria digit*ata, *Fucus serratus*, *Gracilaria gracilis* and *Codium fragile*) are also harvested, and sustainable levels in excess of those currently targeted are likely to be available. Therefore, macroalgae represent an abundant and renewable resource and are therefore being explored as novel sources of compounds with the potential for delaying the onset of certain diseases such as chronic inflammation, atherosclerosis, cancer and cardiovascular disorders (Park et al. 2004). Many inter-tidal species have well-developed antioxidant systems as a defence against reactive oxygen species (ROS) formed as a consequence of dynamic environmental

conditions of their habitat. These ROS are also formed in human cells by endogenous factors, and their deregulation results in oxidative damage which may contribute to the development of a variety of chronic disease states including coronary heart disease, cancer (Reaven and Witzum 1996), diabetes, rheumatoid arthritis (Baverova and Bezek 1999), chronic inflammatory disease of the gastrointestinal tract, Alzheimer disease (Chauhan and Chauhan 2006) and other neurological disorders associated with the ageing processes (Temple 2000). In addition to their potential role in reducing the risk of some diseases, naturally derived antioxidants could be used to control oxidative processes which lead to losses in the quality of processed foods. For example, oxidation of unsaturated lipids leads to the production of rancid flavours and odours while also reducing the shelf-life, nutritional quality and safety of food products (Zainol et al. 2003). Synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been added to many foods to reduce oxidative deterioration. However, due to potential safety issues relating to toxicity (Witschi and Lock 1978), consumers are beginning to show preferences for naturally derived antioxidants.

Whilst a number of previous studies have assessed the antioxidant activity of crude macroalgae extracts from the species under investigation in the present study (Le Tutour 1990; Le Tutour et al. 1998; O'Sullivan et al. 2011), relatively few have attempted to enrich the antioxidant components in these macroalgae species. In their crude form, extracts from macroalgae do not usually contain concentrations of antioxidants at levels comparable to those found in other well-known terrestrial sources of antioxidants such as herbs and spices (Hossain et al. 2008) and some berries and fruits (Heinonen and Meyer 2002) . Therefore, in order for macroalgae to realise their full commercial potential, relatively simple and inexpensive techniques to increase their antioxidant load are essential. Several types of types of antioxidants are found in macroalgae; however phlorotannins, a type of polyphenol unique to seaweeds, have thus far garnered the most attention. Phlorotannins result from the oligomeric dehydrogenative coupling of phloroglucinol (Quideau et al. 2011) and contain both multiple resonance stabilising sites and a range of condensed units which together have the potential to enhance their antioxidant activity. The range of sizes in which phlorotannins can occur varies greatly, and therefore, fractionation techniques which use molecular size as a basis for separation could have a great potential for enriching the antioxidant potential of crude macroalgal extracts. Recently, Tierney et al. (2013) demonstrated that the enrichment of macroalgal phlorotannins from solid-liquid extracts (SLE) of three brown macroalgae (*Fucus spiralis*, *Pelvetia canaliculata* and *Ascophyllum nodosum*) was possible using MWCO dialysis. Therefore, the principal objective of the present study was to examine the use of polarity separation and molecular weight cut-off (MWCO) dialysis as means of enriching crude extracts from a further four species of macroalgae (*L. digitata*, *F. serratus*, *G. gracilis* and *C. fragil*e) based on phenolic content and activity in two in vitro antioxidant assays. Three of these particular species, *L. digitata*, *G. gracilis* and *C. fragile*, are known to have relatively low levels of antioxidant activity in crude extracts (Zhang et al. 2006, 2007; Wang et al. 2009), and it is hoped this approach could enrich extracts from these species with antioxidant compounds. The principal objective of the present study therefore is to investigate if relatively simple and inexpensive techniques could be used to enrich the antioxidant content of crude extracts. This would both increase the commercial potential of underutilised species whilst also providing vital insights into the molecular weight distribution of antioxidant species present in them.

Materials and methods

All solvents used were HPLC grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH·), ferrous chloride, ferrozine, 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), sodium acetate anhydrous, Gallic acid, Folin-Ciocalteu's phenol reagent and sodium carbonate were obtained from Sigma-Aldrich (, Ireland). BioDesignDialysis TubingTM with a 3.5-kDa cut-off was acquired from Fisher Scientific (Ireland). Spectra/Por® Biotech cellulose ester dialysis tubing with a 100-kDa cut-off was obtained from Apex Scientific (Ireland).

Seaweed materials

The four seaweed species investigated in this study *Fucus serratus*, *Gracilaria gracilis*, *Codium fragile* and *Laminaria digitata* were collected from the West coast of Ireland in Co. Clare in spring 2011. A random selection of different plants were taken from the shore, packed in cool boxes and transported immediately to the laboratory. Samples were washed to remove sand and epiphytes and were then stored at −18°C. The taxonomy of all samples was verified by a trained phycologist, and a dried reference sample for each species was stored in NUI Galway as part of the Marine Functional Foods Research Initiative. Samples were freeze-dried then ground into a powder using a Waring blender and stored in vacuum-packed bags at −80 °C prior to extraction.

Preparation of crude seaweed extracts

A range of food-grade solvents were adopted for extraction; however, methanol was also used to prepare crude extracts as to date it has been the solvent of choice for extracting

polyphenols and thus served as a useful comparison to solvents such as water and ethanol. These solvents were previously shown to be effective solvents for the generation of antioxidants from macroalgae (Wang et al. 2009; Ye et al. 2009). Solid-liquid extraction using four different solvent systems, namely cold water (CW), hot water (HW), ethanol/water (80:20; EW) and methanol/water (70:30; MW) was used to prepare the crude extracts. Specifically, extracts were prepared by placing 150 g of the seaweed powder in a conical flask and adding the extraction solvent at a ratio of 10:1 (*v*/*w*) for the methanol- and ethanol-based extracts and a ratio of 20:1 (*v*/*w*) for the cold and hot water extracts. The mixture was then shaken at 150 rpm at room temperature for 24 h; in the case of the hot water extract, the temperature used was 60 °C. The HW extraction was a sequential extraction carried out on the residue from the CW extraction at 60 °C to ensure exhaustive extraction of the seaweed occurred. The EW and MW extractions were filtered three times over a 24-h period through a Buchner funnel, while the CW and HW were filtered twice over a 24-h period through glass wool, and the solvent refreshed each time. Alcohol was removed from EW and MW extracts using a large-scale rotary evaporator (Büchi Rotavapor R-200 with a V710 vacuum pump, Switzerland) water-bath set at 50 °C. The remaining aqueous portions of the alcohol-based extracts were frozen and freeze-dried. The water-based extracts were filtered through glass wool combined with cotton wool to remove particles that were difficult to remove due to their viscous nature. These were then concentrated under a reduced pressure using the large-scale rotary evaporator, frozen at −20 °C and freeze-dried. All extracts were subsequently ground to a fine powder using a mortar and pestle prior to bioactivity testing.

Preparation of molecular weight cut-off and hydrophobic fractions

Prior to dialysis the crude EW and MW extracts were subjected to partitioning exhaustively using water to create a hydrophobic fraction (HPF) (remaining residue) and the hydrophilic fraction (water soluble portion). The crude CW and HW solid-liquid extraction (SLE) extracts and the hydrophilic fraction partitioned from the EW and MW extracts were dissolved in minimal volume of deionised water and decanted into a 3.5-kDa dialysis tubing clamped at one end. The tubing was clamped at the other end, immersed in a reservoir of deionised water and shaken moderately (50 rpm) at room temperature for 72 h. The reservoir of water was refreshed periodically until the no further colour was visible in the dialysate. Both the high molecular weight (HMW) retentate (>3.5 kDa) and low-molecular weight (LMW) dialysate (<3.5 kDa) fractions were freeze-dried. The HMW retentate from the 3.5-kDa dialysis was dissolved in water, placed in a 100-kDa dialysis tubing, and dialysis was carried out as described previously. Both the retentate (>100 kDa) and dialysate (3.5–100 kDa) fractions were freeze-dried. All MWCO fractions and the hydrophobic fractions were then assayed in vitro for total phenolic content (TPC), DPPH radical scavenging activity (RSA), and ferric reducing antioxidant power (FRAP) activity.

Reverse-phase (RP) flash chromatography

The lower molecular weight (<3.5 kDa) fraction of the species *F. serratus* was further fractionated using a two-step reverse-phase (RP) flash chromatography method. RP flash chromatography was carried out on a Varian Intelliflash 310 system using a TELOS^{TM} C18 column with a sorbent mass of 375 g and mean particle size of 40–60 μm. Two gram of the <3.5-kDa fraction material dissolved in water was loaded onto the column. A two-step elution gradient was employed. The mobile phase consisted of the primary eluent of HPLC grade water (0–20 min) and the secondary eluent of 100 % methanol (20–40 min). The flow rate was 50 mL min^{-1}. Flash fractions were collected from 0 to 20 min (flash fr. 1) and from 20–40 min (flash fr. 2). UV detection was observed at 210, 225 and 250 nm. All flash fractions were assayed for TPC, DPPH and FRAP activity.

Assay for antioxidant activities

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed according to Straitil et al. (2006) with minor modifications. This method allows the determination of the ferric-reducing ability (μmol $Fe^{(III)}$ converted to $Fe^{(II)}$ in aqueous solutions) of the samples, as a measure of their antioxidant power. The FRAP reagent contained 10 mL of 10 mmol L^{-1} TPTZ (2,4,6-tri(2-pyridyl-5-triazine) solution in 40 mmol L^{-1} of HCL plus 10 mL of 20 mmol L^{-1} $FeCl_3.6H_2O$ and 100 mL of 0.3 mol L^{-1} acetate buffer, pH 3.6. A 2-mM Trolox stock solution was prepared and diluted with methanol to give concentrations ranging from 0.1–0.4 mM. In brief, 180 μL of freshly prepared FRAP reagent at 37 °C was pipetted into a 96-well micro titre plate with either a 20-μL test sample or standard (or methanol for the blank). Samples were tested at 1 mg mL^{-1}. Samples were incubated at 37 °C for 40 min, and then the absorbance was measured at 595 nm using a plate reader (BMG Labtech FLUOstar Omega, Germany). Trolox was used as a standard, and FRAP values were expressed as microgram trolox equivalents per milligram dry weight sample (μg TE mg^{-1} DW sample). All fractions were tested in triplicate.

DPPH (1,1-Diphenyl-2-picryl-hydrazyl) scavenging activity

The free-radical scavenging capacity of the crude and the fractionated seaweed extracts were analysed using a modified method of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

according to Goupy et al. (1999). A 2-mM Trolox stock solution was prepared and diluted with methanol to give concentrations ranging from 0.1–0.4 mM. This standard curve is used to determine the IC_{50} value of Trolox and to ensure method and solutions are correct. One hundred millilitre of methanol was added to each well apart from well 1. Two hundred microlitre of each sample was pipetted into well 1 of the plate. Serial dilutions of the seaweed samples starting at 2 mg mL^{-1} in well 1 were prepared across a 96-well micro titre plate. One hundred microlitre of a 1 in 5 dilutions of the DPPH/methanol (0.238 mg of DPPH per millilitre of methanol prepared daily) working solution was pipetted into each well. The plate was then placed in the dark at room temperature for 30 min. The absorbance was measured at 515 nm using a plate reader. The decrease in absorbance of the sample extract was calculated by comparison to a control (100 μL sample extraction solvent and 100 μL DPPH·). The relative decrease in absorbance (PI (% inhibition)) was calculated using Eq. 1 below;

$$\mathrm{PI}(\%) = [1-(\mathrm{Ae}/\mathrm{Ab})] \times 100, \quad (1)$$

where Ae=absorbance of sample extract and Ab=absorbance of control. PI's were used to calculate the related antioxidant activity according to the method of Ollanketo et al. (2002) i.e. where PI1 (superior) and PI2 (inferior) were used to estimate the concentration of extract required to result in a 50 % decrease of DPPH absorbance. Antioxidant activity was expressed as antiradical power (ARP), which is the reciprocal of the IC_{50} (mg mL^{-1}) used to define the concentration of a sample extract that produces a 50 % reduction of the DPPH radical absorbance (Ollanketo et al. 2002). High ARP values indicate a strong RSA of a sample (Brand-Williams et al. 1995). All fractions were tested in triplicate.

Determination of total phenolic content

The total phenol content of the extract was quantified according to the method of Singleton et al. (1999). Samples were diluted in methanol and tested at 1 mg mL^{-1}. Briefly, a 100-μL aliquot of sample was mixed with a 100-μL Folin-Ciocalteu's phenol reagent, 100-μL methanol and 700-μL 20 % Na_2CO_3. The reaction mixture was mixed thoroughly and allowed to stand for 20 min at room temperature in the dark. Samples were centrifuged at 13,000 rpm for 3 min, and the absorbance of all samples was measured at 735 nm using a Hitachi U-2900 spectrophotometer. Gallic acid was used as a standard; standards ranging from 10–200 μg mL^{-1} were used to construct a standard curve. Methanol was used for the standard dilution and also used as a blank. The total phenol content was expressed in terms of microgram gallic acid equivalents per milligram of dry weight sample (μg GAE mg^{-1} sample). All fractions were tested in triplicate.

Quadrupole time-of-flight mass spectrometry (Q-Tof-MS)

Mass spectrometry analysis of the LMW polyphenol-enriched flash samples was performed using a Q-Tof Premier mass spectrometer (Waters Corporation, Micromass MA Technologies, UK) by direct infusion into the electrospray ionisation source. Mass spectral data was obtained in the negative ion mode for a mass range of 100 to 3,000 m/z. Capillary and cone voltages were set at 3 and 45 kV, respectively. The desolvation gas was set at 800 L h^{-1} while the cone gas was set at 50 L h^{-1}. Samples were dissolved in methanol, filtered and infused at 10 μL min^{-1} for 2 min.

Statistical analysis

All analyses were performed in triplicate. Results are presented as mean values±SE. The means of all the parameters were examined for significance by analysis of variance (ANOVA) followed by Tukey's test to determine significant differences between species. This was done using the statistical software Minitab15. Significance of differences was defined at the 5 % level ($p<0.05$).

Results

Total phenol content (TPC) of MWCO fractions

Tables 1, 2, 3 and 4 present the antioxidant activity and phenolic contents of crude and enriched extracts. The levels of phenols in the crude algae extracts varied considerably ranging from 0.99 to 81.93 μg GAE mg^{-1} sample in CW extracts from the brown seaweed *F. serratus* and the MW extract in the green seaweed *C. fragile*. Greater than a twofold increase was observed in the 3.5–100-kDa fraction of the methanol/water (MW) extracts (185.13 μg GAE mg^{-1} sample) in *F. serratus* in comparison to the corresponding crude extract (80.70 μg GAE mg^{-1} sample). However, the 100-kDa hot water fraction had a lower phenolic content than the crude extracts along with the 3.5-kDa fractions of all the *F. serratus* extracts. In comparison to *F.serratus*, much lower levels of phenols were observed for *L. digitata* (MW crude= 2.93 μg GAE mg^{-1} sample), *C. fragile* (MW crude= 0.99 μg GAE mg^{-1} sample) and *G. gracilis* (MW crude= 5.36 μg GAE mg^{-1} sample). On average, a 5–6 fold increase in the phenolic content of MWCO fractions was observed compared to the crude fractions. The methanol/water and ethanol/water hydrophobic fractions had the highest phenolic content. For example, for *L. digitata*, the hydrophobic fractions (HPF methanol/water=28.13 μg GAE mg^{-1} sample and HPF ethanol/water=37.00 μg GAE mg^{-1} sample) exhibited a fourfold increase in phenolics in comparison to the crude extracts (MW crude=2.93±0.77 μg GAE mg^{-1} and EW

Table 1 Total phenol content (TPC), ferric reducing antioxidant potential assay (FRAP) and 1,1-diphenyl-2-picryhydrazyl (DPPH·) scavenging assay of crude extracts and enriched fractions from *Fucus serratus*

Extracts	Fraction	TPC (μg GAE mg^{-1} sample)	FRAP (μg TR equivalents mg^{-1} sample)	DPPH (ARP)
MW	Crude	80.70±1.34e	75.55±0.78g,h	7.82±0.17g
MW	<3.5	26.87±0.35h	17.79±0.18°	0.65±0.03j
MW	3.5–100	185.13±1.32a	152.85±1.12d	20.45±0.65d
MW	>100	131.70±0.40b	217.80±5.44c	24.12±0.96c
MW	HPF	91.40±0.56d	42.01±0.01m	2.93±0.06i
EW	Crude	75.96±10.11e,f	69.30±0.85i	18.63±1.46d
EW	<3.5	27.40±0.00h	10.91±0.65p	0.63±0.05j
EW	3.5–100	131.37±0.40b	270.86±1.28b	48.13±1.79a
EW	>100	130.27±0.61b	313.53±1.42a	45.03±0.73b
EW	HPF	117.37±0.45c	44.40±0.00l,m	5.34±0.15h
HW	Crude	79.49±1.09e	89.27±0.80e	12.46±0.53f
HW	<3.5	24.27±0.25h	48.86±0.59k,l	7.85±0.30g
HW	3.5–100	91.27±0.32d	71.67±0.33h,i	19.54±0.77d
HW	>100	39.13±0.35g	24.97±0.29n	11.50±0.09f
CW	Crude	81.93±1.93e	84.37±0.38f	12.72±0.62e,f
CW	<3.5	14.93±0.12i	29.50±0.40n	5.98±0.46g,h
CW	3.5–100	68.50±0.20f	50.22±0.32k	14.94±0.69e
CW	>100	96.60±0.66d	55.03±1.52j	10.68±0.72f

EW ethanol/water extract, *MW* methanol/water extract, *HW* hot water extract, *CW* cold water extract, *HPF* hydrophobic fraction

Values are means±S.D. (n=3)

Column-wise values with different letters (a, b, c etc.) indicate a significant difference (p<0.05)

crude=1.39±0.24 μg GAE mg^{-1} sample). The methanol/water and ethanol/water hydrophobic fractions showed a significant 4–5 fold increase in phenolic content in both *G. gracilis* and *C. fragile*, with a significant increase (p<0.05) also observed in the enriched MWCO fractions in comparison to the crude extracts of *G. gracilis* (MW crude=5.36±0.29 μg GAE mg^{-1} sample) and *C. fragile* (MW crude=0.99±0.11 μg GAE mg^{-1} sample and EW crude=2.40±0.050 μg GAE mg^{-1} sample).

Table 2 Total phenol content (TPC), ferric reducing antioxidant potential assay (FRAP) and 1, 1-diphenyl-2-picryhydrazyl (DPPH·) scavenging assay of crude and enriched fractions from *Laminaria digitata*

Extracts	Fraction	TPC (μg GAE mg^{-1} sample)	FRAP (μg TR equivalents mg^{-1} sample)	DPPH (ARP)
MW	Crude	2.93±0.77k	3.99±0.30h	0.42±0.06e,f
MW	<3.5	13.10±0.00f,g,h	25.90±0.19c	0.27±0.01g,h,i
MW	3.5–100	12.88±0.06g,h	8.04±0.14g	0.96±0.04b
MW	>100	18.60±0.17d	37.02±0.29a	1.53±0.09a
MW	HPF	28.13±0.25b	4.72±0.00h	0.60±0.01d
EW	Crude	1.39±0.24l	8.54±0.27f,g	0.21±0.02i,j
EW	<3.5	12.87±0.06g,h	22.62±0.31d	0.23±0.01i,j
EW	3.5–100	13.67±0.29f,g	13.15±0.37e	0.62±0.00d
EW	>100	16.10±0.20e	21.15±0.35d	0.14±0.01j
EW	HPF	37.00±0.20a	12.23±0.00e	0.67±0.00d
HW	Crude	5.06±0.87j	0.49±0.15i	0.25±0.03h,i
HW	<3.5	11.80±0.00i	9.10±0.06f,g	0.33±0.01f,g,h
HW	3.5–100	19.60±0.10d	33.64±0.49b	0.35±0.01e,f,g
HW	>100	22.27±0.59c	12.54±1.69e	0.33±0.01f,g,h
CW	Crude	2.24±0.22k,l	8.17±0.59g	0.65±0.01d
CW	<3.5	12.60±0.00h,i	9.86±0.46f	0.43±0.01e
CW	3.5–100	13.94±0.06f	25.94±0.43c	0.79±0.02c
CW	>100	14.07±0.06f	22.43±0.21d	0.41±0.01e,f

EW ethanol/water extract, *MW* methanol/water extract, *HW* hot water extract, *CW* cold water extract, *HPF* hydrophobic fraction

Values are means±S.D. (n=3)

Column-wise values with different letters (a, b, c etc.) indicate a significant difference (p<0.05)

Table 3 Total phenol content (TPC), ferric reducing antioxidant potential assay (FRAP) and 1, 1-diphenyl-2-picryhydrazyl (DPPH·) scavenging assay of crude and enriched fractions from *Gracilaria gracilis*

Extracts	Fraction	TPC (μg GAE mg^{-1} sample)	FRAP (μg Tr equivalents mg^{-1} sample)	DPPH ARP
MW	Crude	5.36±0.29i	6.26±0.31m	0.14±0.01i
MW	<3.5	11.97±0.06h	29.60±0.37d	0.49±0.00a
MW	3.5–100	15.13±0.06e	30.40±0.36c,d	0.41±0.01c,d
MW	>100	12.00±0.40h	65.62±0.61a	0.15±0.02h,i
MW	HPF	21.70±0.60b	27.83±0.03e	0.22±0.01g
EW	Crude	4.76±0.17i	4.76±0.16n	0.16±0.00h
EW	<3.5	15.00±0.10e,f	24.43±0.20g	0.42±0.00c
EW	3.5–100	16.00±0.10d	22.43±0.78h	0.46±0.00b
EW	>100	13.57±0.12g	35.17±1.02b	0.27±0.00f
EW	HPF	34.30±0.30a	3.57±0.00n	0.11±0.00j
HW	Crude	3.49±0.30j	7.30±0.08k,l,m	0.22±0.01g
HW	<3.5	17.80±0.10c	9.47±0.06j	0.48±0.01a,b
HW	3.5–100	18.17±0.12c	31.04±0.37c	0.41±0.00c,d
HW	>100	14.53±0.12e,f	25.77±0.55f	0.15±0.01h,i
CW	Crude	4.91±0.27i	10.91±0.19i	0.24±0.01g
CW	<3.5	14.43±0.15e,f	6.47±0.18l,m	0.27±0.01f
CW	3.5–100	16.70±0.10d	7.60±0.32k,l	0.39±0.01d
CW	>100	14.30±0.27f,g	8.19±0.48j,k	0.34±0.00e

EW ethanol/water extract, *MW* methanol/water extract, *HW* hot water extract, *CW* cold water extract, *HPF* hydrophobic fraction

Values are means±S.D. (n=3)

Column-wise values with different letters (a, b, c etc.) indicate a significant difference ($p<0.05$)

DPPH radical scavenging activity

Fucus serratus extracts were the most effective scavengers of DPPH radicals. From the crude extracts from *F. serratus*, the EW extract had the greatest scavenging ability with and ARP value of 18.63 and the MW of *G. gracilis* had the lowest scavenging ability with an ARP value of 0.14. All the *F. serratus* crude extracts showed a good radical scavenging

Table 4 Total phenol content (TPC), ferric reducing antioxidant potential assay (FRAP) and 1, 1-diphenyl-2-picryhydrazyl (DPPH·) scavenging assay of crude and enriched fractions from *Codium fragile*

Extracts	Fraction	TPC (μg GAE mg^{-1} sample)	FRAP (μg Tr equivalents mg^{-1} sample)	DPPH ARP
MW	Crude	0.99±0.11h	3.44±0.29h	0.16±0.01h,i
MW	<3.5	12.70±0.10f	19.29±0.26c	0.52±0.01a
MW	3.5–100	12.57±0.06f	4.79±0.15g	0.39±0.01b,c
MW	>100	12.90±0.00e,f	13.63±0.28e	0.21±0.02f,g
MW	HPF	30.70±0.20b	13.24±0.00e	0.12±0.02i,j
EW	Crude	2.40±0.50h	6.01±0.29g	0.17±0.01g,h,i
EW	<3.5	12.70±0.00f	10.40±0.19f	0.35±0.01c,d
EW	3.5–100	12.33±0.06f	5.84±0.27g	0.27±0.00e,f
EW	>100	16.57±0.06c	19.89±0.38c	0.13±0.01i,j
EW	HPF	34.87±0.06a	10.47±0.00f	0.10±0.05j
HW	Crude	7.89±0.14g	0.94±0.03i	0.37±0.03c,d
HW	<3.5	15.47±0.84d	32.37±0.25a	0.21±0.02f,g
HW	3.5–100	13.63±0.06e	17.63±0.19d	0.33±0.00d
HW	>100	15.70±0.10d	32.70±0.10a	0.24±0.01e,f
CW	Crude	3.23±0.12h	2.77±0.12h	0.56±0.01a
CW	<3.5	12.33±0.12f	22.53±0.25b	0.18±0.01g,h
CW	3.5–100	13.67±0.06e	17.97±0.23d	0.42±0.01b
CW	>100	17.27±0.06c	32.19±1.40a	0.17±0.01g,h,i

EW ethanol/water extract, *MW* methanol/water extract, *HW* hot water extract, *CW* cold water extract, *HPF* hydrophobic fraction

Values are means±S.D. (*n*=3)

Column-wise values with different letters (a, b, c etc.) indicate a significant difference ($p<0.05$)

ability in comparison to the other species. The CW and MW crude extracts of *L. digitata* had a low scavenging ability with an ARP value of 0.65 and 0.42, respectively. Low scavenging ability was also observed in the crude CW and HW extracts of *C. fragile* with an ARP value of 0.56 and 0.37, respectively. A very low scavenging ability was observed in all crude extracts of *G. gracilis* (MW=0.4±0.01; CW=0.24±0.01). Similar to the observation for phenolic contents, a higher activity was observed in the MWCO-enriched fractions in comparison to the crude extracts. For example, a significant enrichment in MWCO fractions was observed in the 3.5–100-kDa (ARP=20.448), >100-kDa (ARP=24.12) of the MW, 3.5–100-kDa (ARP=48.128) and the >100-kDa (ARP=45.033) (EW) fractions from *F. serratus*. This is a significant increase ($p<0.05$) in comparison to the crude extracts, MW crude (ARP=7.815) and EW crude (ARP=18.633). In particular, the 3.5–100-kDa EW fraction had an ARP value circa 2.5 times greater than the crude extract, and the >100-kDa MW fraction had an ARP 3 times greater than the crude fraction. Decreased activity was observed in the <3.5-kDa fraction and the hydrophobic fractions for the same extracts. With respect to the aqueous extracts of *F. serratus*, a significant increase ($p<0.05$) in activity in comparison to crude extracts was only observed in the >100-kDa fraction of the hot water (HW) and cold water (CW) extracts. In fact, a decrease in activity in the <3.5-kDa and 3.5–100-kDa fractions was observed for these extracts in comparison to the crude extract. This implies that for aqueous extracts, higher molecular weight compounds are the principal components responsible for the radical scavenging ability of *F. serratus*. For *L. digitata*, a twofold increase in activity was observed in the 3.5–100 kDa (ARP=0.96) and a 3.5 fold increase in the >100-kDa (ARP=1.53) fractions of the MW in comparison to crude extracts (MW crude ARP=0.42); a slight increase was also observed in the hydrophobic fraction, but a decrease in radical scavenging activity was obtained for the <3.5-kDa fraction of the MW extract in comparison to the crude extract.

Ferric reducing antioxidant power (FRAP)

The level of FRAP activity differed quite significantly between species. A significant increase ($p<0.05$) in particular was observed in the 3.5–100-kDa and >100-kDa methanol and ethanol extracts of *F. serratus* in comparison to crude extracts (MW crude=75.55 μg TE mg^{-1} sample and EW crude=69.30 μg TE mg^{-1} sample). However, a reduction in FRAP activity was observed in the hot and cold water extracts of *F. serratus*. This is also seen in the cold water extracts (<3.5 kDa=6.47, 3.5–100 kDa=7.60 and >100 kDa=8.19) of *G. gracilis*. Enrichment increased FRAP levels comparable with species with a good antioxidant activity. This is seen in the MW >100-kDa fraction of *G. gracilis* (65.62 μg TE mg^{-1} sample) which exhibits a similar FRAP activity to the species *A. nodosum* ethanol extract (66.08 μg TE mg^{-1} sample) from the study by Tierney et al. (2013).

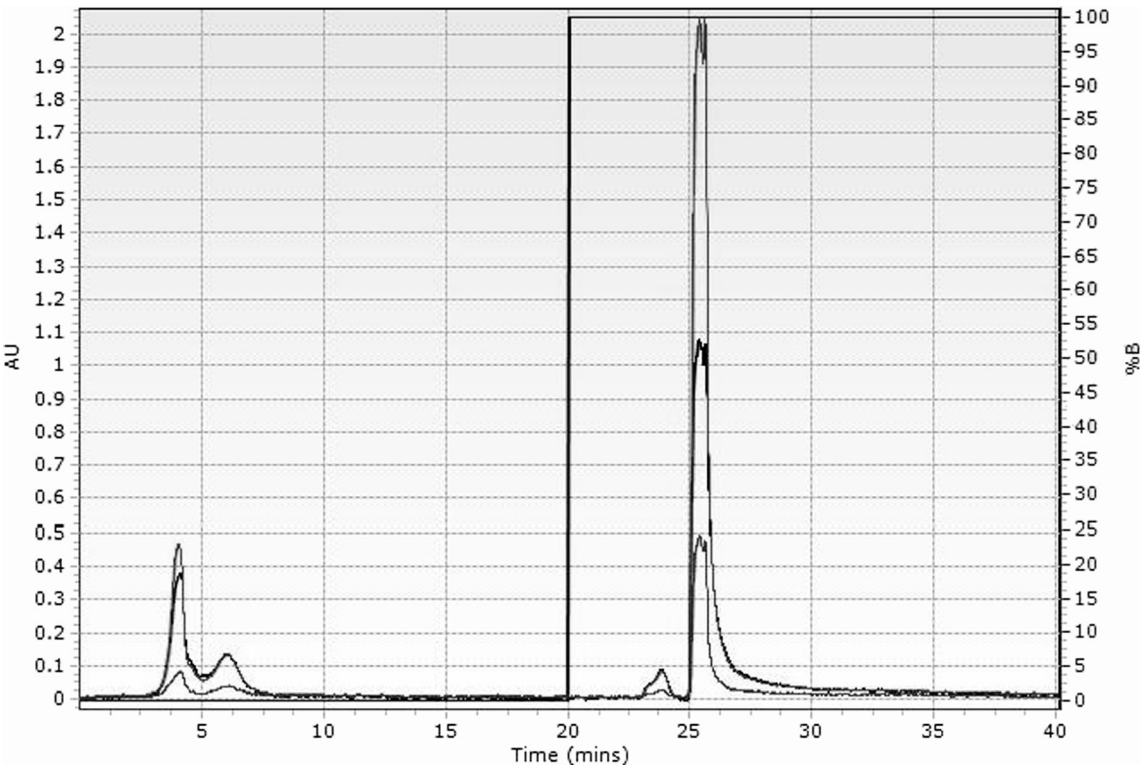

Fig. 1 Flash chromatogram of *Fucus serratus* cold water extract. First set of peaks are sugars and the second set represent phlorotannins

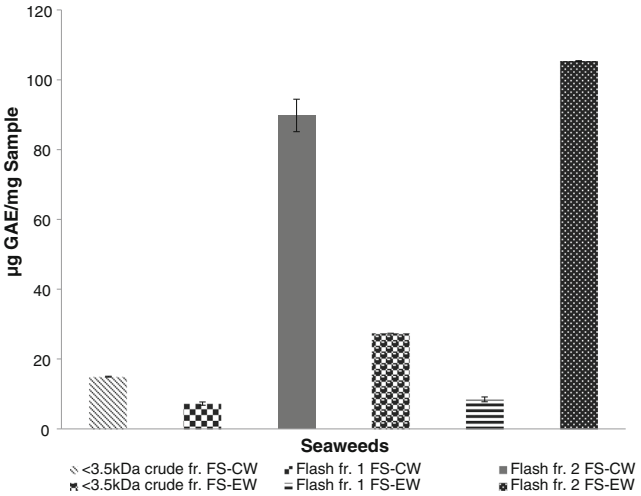

Fig. 2 Total phenolic content (TPC) expressed as microgram gallic acid equivalents (GAE) of a less than 3.5-kDa molecular weight cut-off fraction and two flash chromatography fractions from the seaweed *Fucus serratus* extracted with two different solvents. Values are means±S.D. (n=3)

Phlorotannin enrichment of low-molecular weight fractions of *Fucus serratus*

The flash chromatograms for both the CW and EW extracts showed an initial peak, detected at 4–10 min which is likely to be very polar compounds, most probably simple sugars and sugar alcohols (Fig. 1). A second peak was detected by UV at 25–35 min in both chromatograms which indicates the presence of predominant polyphenols (phlorotannins) in this fraction based on previous reports (Tierney et al. 2013).

Total phenolic content (TPC) and in vitro antioxidant activity of phlorotannin-enriched RP flash fractions

More than a threefold enhancement in the TPC was observed in the flash fr. 2 for the ethanol/water fraction (FS-EW) (105.38 µg GAE mg^{-1} sample) relative to the less than 3.5-kDa fraction of the extracts (27.4 µg GAE mg^{-1} sample) (Fig. 2), this is presumably due to the removal of low-molecular weight compounds such as sugars from this fraction. A decrease in TPC activity was seen in flash fr. 1. For the cold water fraction (FS-CW), a considerable sixfold increase was seen in the enriched flash fr.2 (89.78 µg GAE mg^{-1} sample) compared to the crude <3.5-kDa fraction (14.93 µg GAE mg^{-1} sample). The highest TPC activity of 105.38 µg GAE mg^{-1} sample was observed in the flash fr. 2 for the ethanol/water (FS-EW) extract. A decrease in activity was seen in flash fr.1 for both the CW and the ethanol/water (EW) extracts in comparison to the 3.5–100-kDa crude fraction. It is most probable that the presence of large amounts of sugar-based compounds in this fraction may have been responsible for masking the activity of the phlorotannins in the crude <3.5-kDa fraction.

Similar to the TPC results, the flash fr. 2 for the ethanol/water extract (FS-EW) exhibited the highest ARP value of 51.984. This is an increase in activity in comparison to the >3.5-kDa crude fraction (ARP=5.975). This fraction also had the highest FRAP activity (69.01 µg TE mg^{-1} sample). An increase in activity was also seen in the cold water (FS-CW) flash fr. 2 in both the DPPH and FRAP assays in comparison to the <3.5-kDa crude fraction.

Discussion

Moderately polar solvents are usually the solvent of choice when the target compounds are polar antioxidants such as polyphenols and tannins, (Cho et al. 2007). In the present study, methanol (70 %), ethanol (80 %), cold water and hot water were used to extract antioxidant compounds from four macroalgae species harvested from Irish shores. Therefore, in the present study, a range of polar solvent systems were selected to generate initial crude extracts. In general, food-grade solvents were used; however, methanol was also used to prepare crude extracts as to date it has been the solvent of choice for extracting polyphenols and thus served as a useful comparison. However, the main objective of the study was to utilise seaweed species that generally would not be considered for bioactive compounds, to pinpoint molecular weight fractions within the seaweed extracts with the potential to exploit, to develop a profiling method to explore these bioactive compounds and to examine the feasibility of using simple and low-cost enrichment techniques based on polarity partitioning and MWCO dialysis to generate three molecular weight fractions (<3.5 kDA, 3.5–100 kDa and >100 kDa) along with a hydrophobic fraction.

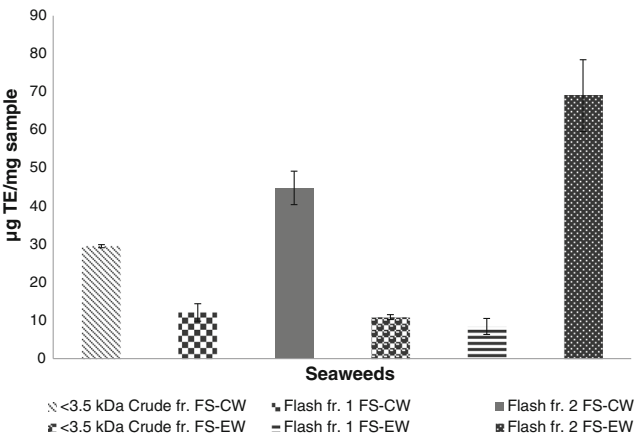

Fig. 3 Ferric reducing antioxidant power (FRAP) expressed as microgram trolox equivalents (TE) of a less than 3.5-kDa molecular weight cut-off fraction and two flash chromatography fractions from the seaweed *Fucus serratus* extracted with two different solvents. Values are means± S.D. (n=3)

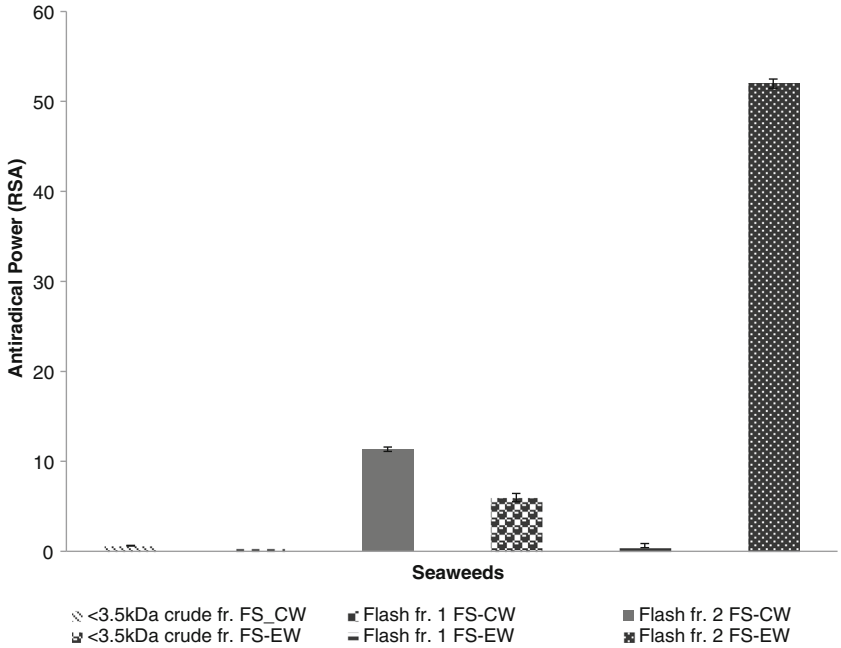

Fig. 4 DPPH radical scavenging ability (RSA) expressed as antiradical power (ARP) of a less than 3.5-kDa molecular weight cut-off fraction and two flash chromatography fractions from the seaweed *Fucus serratus* extracted with two different solvents. Values are means±S.D. (n=3)

Tables 1, 2, 3 and 4 present the antioxidant activity and phenolic contents of crude and enriched extracts of the four macroalgal species, and the proceeding sections will discuss the success of the enrichment approach in relation to antioxidant activity (FRAP and DPPH activity) and phenolic content for each of the selected macroalgae species.

The TPC of crude and enriched fractions from the four seaweed species could be ranked in the following order of decreasing total phenolic content, *F. serratus*, *L. digitata*, *G. gracilis* and *C. fragile*. This variation in part is a natural consequence of the type of seaweed under investigation. In particular, brown macroalgae are reported to generally contain higher amounts of polyphenols than red and green algae (Zubia et al. 2007). Wang et al. (2009) have also reported high levels of TPC in extracts of *F. serratus*, a high TPC was observed in the 70 % acetone (24.0 g PGE (100 g)$^{-1}$) and water (16.9 g PGE (100 g)$^{-1}$) extracts of this species. MWCO fractionation of the crude extracts resulted in a significant increase (p<0.05) in phenolic content in nearly all 3.5–100-kDa, >100-kDa and hydrophobic fractions from *F. serratus* in comparison to the crude extracts (Table 1). Following fractionation, a significant increase (p<0.05) in phenolic content was observed in all MWCO fractions of *L. digitata*, *G. gracilis* and *C. fragile* in comparison to the crude extracts for the same species. The enrichment process using MWCO enhanced the activity of sample fractions to levels comparable with crude extracts from other macroalgae species that are considered to have good activity. For example, in the species *C. fragile*, the MW-HPF and the EW-HPF exhibited a TPC activity as high as the ethanol extract from the species *Ulva Intestinalis* (41.40 μg PE mg^{-1} sample) in the study of Tierney et al. (2013).

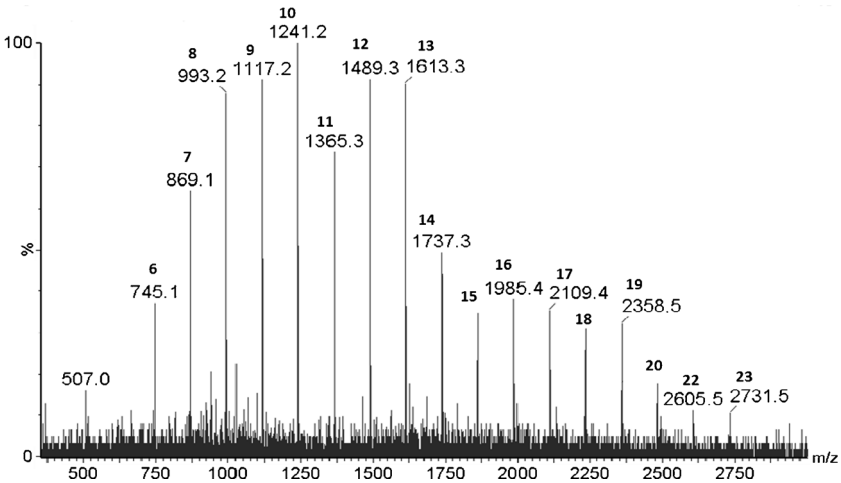

Fig. 5 ESI-Q-ToF-MS spectrum showing the distribution of molecular weights of molecules in *Fucus serratus* ethanol/water (FS-EW). (Number on top represent no. of phloroglucinol units)

The DPPH activity is expressed as the antiradical power (ARP) which is the reciprocal of the IC50 (mg mL^{-1}) and defined as the concentration of sample extract that produces a 50 % reduction of the DPPH radical absorbance (Ollanketo et al. 2002). All the *F. serratus* crude extracts showed a good radical scavenging ability in comparison to the other species. Similar to the results observed, Jiménez-Escrig et al. (2001) also reported that brown seaweed *Fucus vesiculosus*, *Laminaria ochroleuca* and *Undaria pinnatifida* generally exhibited a better DPPH scavenging capacity than the red seaweeds *Chondrus crispus* and *Porphyra umbilicalis*. The authors also reported that the highest scavenging activity was observed in the *Fucus* species, and no activity was detected for *C. crispus*. Wang et al. (2009) also reported a high scavenging activity (ARP=90.8) in the acetone extract of the species *F. serratus*, while *L. digitata* had a low scavenging activity for both the acetone and water extracts. Work done by Yilmaz Koz et al. (2009) also reported that *C. fragile* exhibited no antioxidant activity when assessed using the DPPH assay. *G. gracilis* also showed a low scavenging capability (3.62 %) when assessed by Zhang et al. (2007). Similar to the observation for phenolic contents, higher activity was observed in the MWCO-enriched fractions in comparison to the crude extracts. In the EW, CW and HW extracts of *L. digitata*, a moderate increase was observed in the 3.5–100-kDa fraction in comparison to the crude extract. Increased radical scavenging activity was observed in most of the MWCO fractions of both *C. fragile* and *G. gracilis* in comparison to their crude extracts.

The level of FRAP activity differed quite significantly between species. *F. serratus* was the only seaweed species that exhibited appreciable FRAP activity, the other three species e.g., *L. digitata*, *G. gracilis* and *C. fragile* had a low FRAP activity. However, similar to the effect outlined for the DPPH assay above, a significant increase ($p<0.05$) was observed in the higher molecular weight fractions of each extract. Comparable to results observed for the TPC, enrichment proved to enhance some sample fractions to levels as good as crude extracts of other macroalgae species with a good antioxidant activity. In agreement with previous studies by O'Sullivan et al. (2011) and Jiménez-Escrig et al. (2001), the *Fucus* species exhibited the highest FRAP activity. Again, it appears that this activity may be attributed to their phlorotannin content. Little work has been done on *F. serratus* to fractionate and identify the molecular regions responsible for the observed activity; however, Tierney et al. (2013) and Wang et al. (2012) investigated the antioxidant activities of molecular weight-fractionated extracts from other brown macroalgae such as *A. nodosum*, *F. spiralis*, *P. canaliculata* and *F. vesiculosus*. Tierney et al. (2013) reported similar results where a significant increase in activity was observed in the HMW fractions (3.5–100 kDa and >100 kDa) with *F. spiralis* EW 3.5–100 kDa reported to have a very high FRAP activity of 559.96 μg TE mg^{-1} sample.

In summary, the results indicated that MWCO fractionation of the seaweed extracts using dialysis tubing of MWCO 3.5 and 100 kDa was an effective tool for significantly increasing the level of antioxidant activity in comparison to crude extracts from the marcroalgae species examined in the present study. In particular, for species that would generally be considered to have low levels of antioxidant activity, enrichment proved to enhance the activity to levels comparable with crude extracts from other macroalgae species that are considered to have a good antioxidant activity. In effect, these enrichment methods have resulted in fractions with high antioxidant activity and/or phenolic content being obtained from species with low activity that are comparable to crude extracts from macroalgal with known high antioxidant content, this is demonstrated in the *C. fragile* hydrophobic fraction in the TPC assay and the MW >100-kDa fraction of *G. gracilis* in the FRAP assay. Kuda et al. (2006) recently showed that the >5-kDa fraction from species *F. serratus* had the highest DPPH scavenging activity and the highest phenolic content. This report and a study by Tierney et al. (2013) are in agreement with the antioxidant activities and phenolic contents reported in Tables 1, 2, 3 and 4 where an increase in activity is observed in mainly the higher molecular weight fractions. Previous reports by Wang et al. (2009) and Tierney et al. (2013) have also observed that the extracts containing high levels of TPC were also potent DPPH radical scavengers, thus suggesting that algal polyphenols may be the principal constituents responsible for the antiradical properties of the extracts. The high antioxidant and phenolic activities observed in the species *F. serratus* may be due to the high presence of phlorotannins that are found in various brown algae species. Studies reported by Tierney et al. (2013) and Wang et al. (2012) have highlighted high antioxidant activities and high phenolic contents in *Fucus* species such as *F. spiralis* and *F. vesiculosus* and have attributed these high activities to the presence of phlorotannins.

Whilst TPC, DPPH and FRAP activities of the <3.5-kDa fractions were considerably lower than those from the 3.5–100 kDa and >100 kDa for all seaweed species, only the brown macroalga *F. serratus* exhibited substantial total phenolic content and antioxidant activity. Therefore, further enrichment of the <3.5 kDa was only pursued for MWCO fractions. Further purification of the EW and CW fractions from this species was carried out to investigate the potential presence of low-molecular weight fractions. Flash chromatography was employed to enrich the phenolic compounds in the <3.5-kDa fractions. Water and methanol were used in a two-step gradient to produce two fractions, a polar fraction and a phlorotannin-enriched fraction. This enrichment process, it is not suitable for use in food products (uses methanol- and silica-based stationary phase); however, it can be applied to

enrich fractions for both pharmaceutical and cosmetic use. Flash fractions were collected at 0–20 min (flash fr. 1) and from 20–40 min (flash fr. 2).

A threefold enhancement in the TPC was observed in the flash fr. 2 for the ethanol/water fraction (FS-EW) relative to the 3.5-kDa fraction of the extracts. This is presumably due to the removal of low-molecular weight compounds such as sugar from this fraction. A decrease in activity was seen in flash fr. 1 for both the CW and EW extracts in comparison to the 3.5–100-kDa crude fraction. Based on solvent conditions and the early elution time, this fraction would presumably contain a high proportion of LMW saccharides.

Similar results to those seen for the TPC values of the enriched flash fractions are also seen in the DPPH and FRAP data (Figs. 3 and 4). Similar to the results seen in the TPC assay, there was considerable decrease in activity seen in flash fr. 1 for both extracts. This would suggest that the flash fr. 2 contains high amounts of low-molecular weight phlorotannins. Comparable results were reported by Tierney et al. (2013), an increase in TPC, DPPH and FRAP activities was seen in flash fractions from the <3.5-kDa crude extract due to the presence of low-molecular weight phlorotannins in three brown seaweed, *A. nodosum*, *P. canaliculata* and *F. spiralis*.

Although flash chromatography is an effective technique for the enhancement of the antioxidant and phenolic activities of the macroalgae extracts, it is not a practical application to foods due to its expense and also because it is not a food-friendly method. However, it was successfully employed to enrich the phenolic compounds in the LMW fractions of *F. serratus* and demonstrates the presence of bioactive seaweed polyphenols in this fraction.

Q-Tof-MS was employed to determine the presence of phlorotannins in the <3.5-kDa fractions of *F. serratus* cold water (CW) and ethanol/water (EW) extracts. The mass spectrum for FS-EW (fr. 2) in the negative ion mode can be seen in Fig. 5. The peak signals have been numbered; these numbers correspond to the degree of polymerization for each phlorotannin peak. The phloroglucinol polymerization ranges 6 to 23 phloroglucinol units (PGU) with the most abundant phlorotannins containing between 8 and 13 phloroglucinol units (PGU) (m/z 993.2 to 1613.3). To the best of our knowledge, this is the first study confirming the presence and degree of polymerization of phlorotannins in *F. serratus*. A number of studies have previously reported the profiling of phlorotannins from other *Fucus* species such as *F. vesiculosus* (Steevensz et al. 2011) and *F. spiralis* (Tierney et al. 2013). The profile of *F. serratus* reported here is somewhat different to *F. spiralis* where the main abundant phlorotannins were in the region of 5 to 8 phloroglucinol units (m/z 621.1 to 993.2.) compared to 8 to 13 PGU for *F. serratus*. This study has provided a great insight into the phenolic content and antioxidant activities of both low-molecular weight and high-molecular weight fractions of this species, the MS data indicating that phlorotannins are the main component for this observed activity from this brown macroalgae.

In conclusion, for *F. serratus* whose crude extracts had the highest activity in all assays, the 3.5-100 kDa of the ethanol extract (EW) had the highest phenolic content of all food-grade fractions. In addition, the >100-kDa fraction of the ethanol extract (EE) of *F. serratus* exhibited the highest FRAP activity, and the 3.5-100-kDa fraction of the ethanol extract of *F. serratus* had the highest DPPH activity. These observations highlight the significant enrichment achievable for extracts from *F. serratus*, which, considering its appreciable antioxidant activity and phenolic content, could have implication with respect to their use as an antioxidant-rich functional food ingredient or in the pharmaceutical and cosmetic industries. Other species examined in the present study initially exhibited very low levels of antioxidant at the crude extract level. MWCO dialysis was a useful low-cost tool for enriching the antioxidant activity and phenolic content of crude extracts from macroalgae with many of the fractions showing greater than threefold enrichment increases following fractionation. In several cases, these fractions are now comparable with the antioxidant activity of species generally considered to have good antioxidant activity. In general, higher molecular cut-off fractions had higher antioxidant activities and phenolic content indicating that the main species responsible for antioxidant activity were most likely to be high-molecular weight phlorotannins. In the case of *F. serratus*, further enrichment of the <3.5-kDa fraction using RP flash chromatography enhanced the antioxidant activity and total phenolic content of the extract by removing highly polar compounds, most likely carbohydrates. The employment of Q-Tof-MS supported the hypothesis that phlorotannins were present in the sample, and the high activity observed in DPPH, FRAP and TPC were due to this group of compounds. The activity seen in the high-molecular weight fractions are presumably due to HMW phlorotannins, which warrants further investigation. The study provides an interesting insight into the possibility of further utilisation of seaweed species that are generally underutilised and the simplicity and low cost of MWCO dialysis to increase the antioxidant activity and phenolic content of crude extracts from macroalgae These techniques can also be scaled up and require a relatively low capital investment. The development of this methodology will assist in the isolation and further detailed analyses of spatial and temporal variabilities of phlorotannins in macroalgae. This in tandem with their associated bioactivity will prove invaluable for future targeted applications of seaweed phenolics.

Acknowledgments The Marine Functional Foods Research Initiative (NutraMara project) is a programme for marine-based functional food development established by the Marine Institute and the Department of Agriculture, Fisheries and Food (DAFF). It is supported by funds

provided under the Strategy for Science, Technology and Innovation 2006–2013 (SSTI) and the Food Institutional Research Measure (FIRM), to establish a Marine Functional Foods Research Programme.

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