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A comparative assessment of the activity and structure of phlorotannins from the brown seaweed *Carpophyllum flexuosum*

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ABSTRACT

The extraction and antioxidant activity of phlorotannins from the brown seaweeds *Carpophyllum flexuosum, Carpophyllum plumosum* and *Ecklonia radiata* was investigated to identify an optimised extraction process for novel anti-oxidant extracts. Subsequently, the composition of the most active phlorotannin extracts was determined. Microwave assisted extraction (MAE) using water was the most efficient extraction process with shorter processing times and a higher purity product than obtained with any of the other methods tested. MAE resulted in the fast, effective decomposition of the cellular structure, as identified through scanning electron microscopy (SEM), and this related directly to the efficiency of extraction. Phlorotannins extracted from *C. flexuosum* by MAE had the strongest antioxidant activity (62.1 mg gallic acid equivalents (GAE)/g dw of seaweed) and more than 5.5-fold greater 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability than ascorbic acid after 7-day incubation periods at 30 °C and at 60 °C. Six major chemical species of phlorotannin, belonging to the fuhalol group, were identified within the MAE extract using NMR and HPLC-MS. The results confirm phlorotannins from *C. flexuosum* to be promising natural, bio-derived and bio-compatible antioxidants, while identifying the most effective method to extract the constituents and retain antioxidant activity.

1. Introduction

Marine macroalgae, or seaweeds, contain a rich diversity of biomolecules (*e.g.*, sulfated carbohydrates, polyphenols, proteins, vitamins, and pigments) with associated bioactive properties and, therefore, have strong potential as a feedstock for functional ingredients for food, pharmaceuticals and cosmeceuticals [1–7]. While lignocellulosic feedstocks are usually difficult to depolymerise due to their inherent structural durability, imparted by their lignin content [8], the vast majority of species of seaweed, especially the brown seaweeds (Phaeophyceae), do not contain any lignin [9,10]. Therefore, due to their comparatively "soft" structure, it is easier to obtain biomolecules from seaweed through physico-chemical fractionation [8]. The production of functional ingredients and high value chemicals from seaweed is also attractive because they do not compete with food crops for land and they are technically simple to grow and harvest [11].

Seaweeds live in an environment of periodical high environmental stress where they are exposed to fluctuating temperatures, increased UV radiation, and high oxygen concentrations [12]. These conditions can easily lead to the formation of strong oxidising agents [13] able to induce cellular damage. However, such damage is not observed *in vivo* [4]. To protect themselves from these environmental stressors, seaweeds produce substances characterised by a strong antioxidant activity such as polyphenols, pigments, sterols, and mycosporine-like amino acids [4,6,14]. Brown seaweeds, in particular, produce a diversity of polyphenolic compounds through the acetate–malonate pathway [15]. These are known as phlorotannins [16] and can constitute up to 25% of dry weight [17–19]. Phlorotannins are comprised of many different molecular structures in which phloroglucinol (1, 3, 5-benzenetriol) monomeric units are combined *via* different linkages into oligomers. The phlorotannins can be categorised into six main groups according to these linkages and to the number of additional hydroxyl groups. Examples of these groups as phlorethols, fucols, fuhalols, fucophlorethols, isofuhalols, and eckols are given in Fig. 1 [20]. Phlorotannins have several biological activities, including antioxidant [21], antibacterial [22] and antiproliferative [23] activity, and the ability to chelate metal ions [24]. Moreover, phloroglucinol, the monomeric unit of phlorotannins, is also a very versatile chemical building block. For example,

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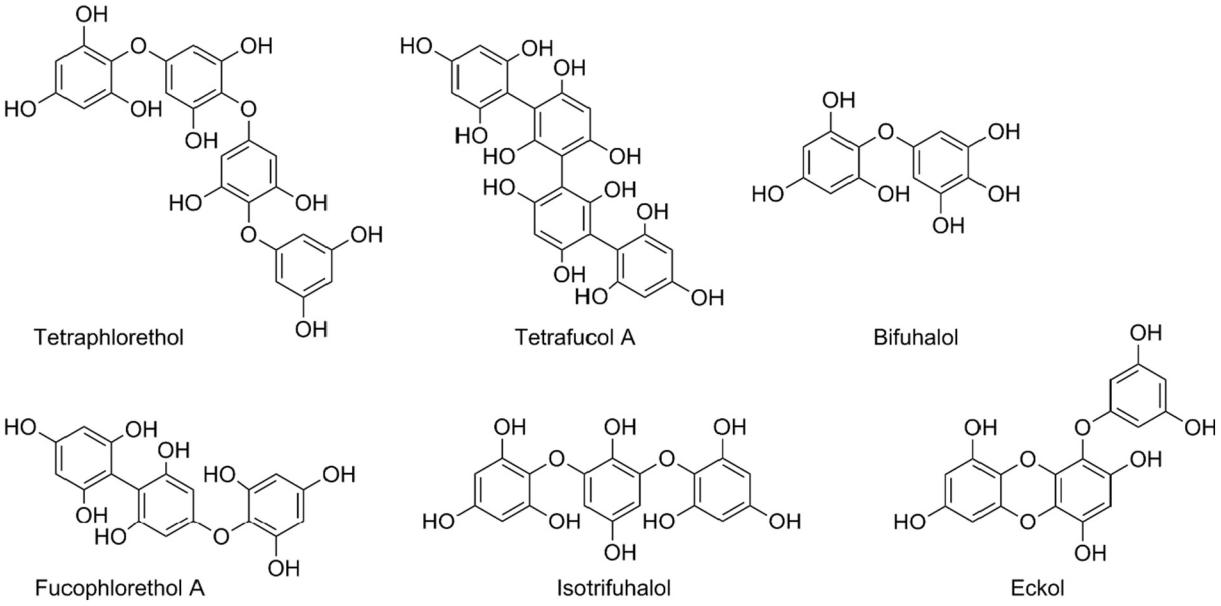

Fig. 1. Example structures of the 6 main classes of phlorotannins.

it can be used to make biocompatible adhesives for tissue adhesion [25] or to form hyperbranched polymers in replacements for Bisphenol A (BPA) [26,27].

Phlorotannins are typically extracted by using solid liquid extraction (SLE) with large volumes of organic solvents such as methanol and acetone with a long extraction time at room temperature or under heating in the presence of base. Within these extraction regimes, significant effort has been made to optimise the extraction parameters [28–30]. Recently, several novel techniques, such as pressurized liquid extraction [31–33]; supercritical fluid extraction [34–36]; ultrasound-assisted extraction [37–39] and microwave assisted extraction [40], have been developed for the extraction of phlorotannins. Underpinning these innovations is the need to improve several aspects of conventional solid–liquid extraction, such as reducing the volume of organic solvent consumed, decreasing extraction time and improving low extraction selectivity. Our group has successfully extracted phlorotannins from *Carpophyllum flexuosum*, *Carpophyllum plumosum* and *Ecklonia radiata* using microwave assisted extraction (MAE), which was demonstrated to be a highly efficient method as compared to conventional SLE. Notably, the extraction yield for *C. flexuosum* was increased by 70% using MAE [41].

In the current study, we first compared methods for the extraction of phlorotannins from 3 species of brown seaweeds to investigate the effect of solvent and pressure on the efficiency of extraction. Changes to the cell morphology of milled, dried seaweed and seaweed residue (post-extraction) were visualised using scanning electron microscopy (SEM) and then related to extraction efficiency. Second, we compared the antioxidant activity of the phlorotannins extracted by the most efficient process (MAE), using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability and ferric-reducing antioxidant power (FRAP). Their thermal stability was compared to that of ascorbic acid. Third, the phlorotannins in the extracts (MAE and SLE) of the most active seaweed, *C. flexuosum*, were purified through liquid–liquid extraction and the compositions of the purified materials were analysed by ^{1}H NMR. Individual phlorotannin structures were identified by means of HPLC-MS.

2. Materials and methods

2.1. Brown algae

The same three species of brown seaweeds: *Carpophyllum flexuosum*, *Carpophyllum plumosum*, *Ecklonia radiata*, as in our previous study comparing MAE and SLE for the extraction of phlorotannins [41] were used (see Magnusson et al. for further details on species selection and collection [41]). The seaweeds were washed carefully with fresh water to remove debris, epiphytes and fauna before being oven-dried at 60 °C to a maximum of 10% internal moisture. They were then milled into fine powder and passed through a 1.0 mm sieve. The dried, powdered seaweed was stored at − 20 °C in sealed bags.

2.2. Chemicals

All chemicals were used as received without further purification except phloroglucinol (Merck Australia), which was recrystallised from water before use to give crystals of its dihydrate. Gallic acid, sodium carbonate, potassium ferricyanide and trichloroacetic acid were purchased from Merck Australia. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, and formic acid were purchased from Sigma-Aldrich, Australia. Iron(III) chloride, monopotassium phosphate, and dipotassium phosphate were purchased from Ajax Fine Chemicals. The Folin-Ciocalteu reagent was purchased from Labchem, Australia. Dichloromethane (Ajax), ethyl acetate and ethanol (Merck) were reagent grade. Acetonitrile was HPLC grade (Merck).

2.3. Extraction procedure

An overview of all the extractions performed in this work, is detailed in the Supporting information, Scheme S1. In order to have comparable results, all these extractions were done within 6 weeks of each other.

2.3.1. Microwave assisted extraction (MAE)

For the total phenolic content assay (TPC), described below in Section 2.5.1, our previously published MAE data are reported here. For all other assays, new extracts produced through MAE conducted as described below were used.

A 500 mg sample of dried, milled biomass from each species of

seaweed was extracted with 15 mL Milli-Q water (Elix® MILLIPORE) in a focused microwave reactor (Anton Paar Monowave 300), heating at 160 °C for 3 min as described in Magnusson et al. [41]. After extraction, the solid residue was removed by filtration and oven-dried at 60 °C for 48 h. The collected filtrate was diluted to 50 mL in a volumetric flask and stored at 4 °C until antioxidant activity assays were performed (details in Section 2.5). All assays were conducted within 24 h of extraction. For thermal stability tests (see Section 2.6), NMR (see Section 2.8) and HPLC-MS analyses (see Section 2.9), the crude extract was freeze-dried (LABCONCO® Freezone 6). The resulting solid residue was weighed and stored at − 20 °C in a tightly sealed container until analysis or further purification (see Section 2.8). Subsamples of each species were extracted 3 times, each on a different day.

2.3.2. Conventional solid-liquid extraction (SLE)

For the total phenolic content assay (TPC), described below in Section 2.5.1, our previously published SLE data are reported here. For all other assays, new extracts produced through SLE conducted as described below were used.

Conventional SLE was performed according the procedure of Magnusson et al. [41]. In brief, a 500 mg sample of dried, milled biomass from each species of seaweed was extracted sequentially with 15 mL volumes of solvent mixture: acidic methanol/water (1:1; acidified to pH 2 with 2 M HCl), followed by acetone/water (7:3, acidified to pH 2 with 2 M HCl) to extract phlorotannins from *C. flexuosum*. The supernatant extract was separated from the solid residue by filtration. Organic solvents in the supernatant were removed at room temperature under reduced pressure and the remaining water was removed by freeze-drying. The resulting solid residue was weighed and stored at − 20 °C in a tightly sealed container before further purification (see Section 2.8). The solid algal residue was washed with Milli-Q water and oven-dried at 60 °C for 48 h. Sub-samples of each species were extracted 3 times, each on a different day.

2.3.3. Room temperature aqueous extraction (RTE)

A 500 mg sample of dried, milled seaweed (*C. flexuosum*, *C. plumosum*, or *E. radiata*) and 15 mL Milli-Q water was stirred at 1200 rpm for 10 min at room temperature. After extraction, the resulting liquid was separated immediately by filtration and then diluted to 50 mL in a volumetric flask. The crude extract was used to measure the total phenolic content by means of the Folin-Ciocalteu assay [41]. The solid algal residue was oven-dried at 60 °C for 48 h. Sub-samples of each species were extracted 3 times, each on a different day.

2.3.4. Conventional hydrothermal extraction (CHE)

A 500 mg sample of dried, milled seaweed (*C. flexuosum*, *C. plumosum*, or *E. radiata*) was extracted with 15 mL Milli-Q water in a 316-stainless steel pressure reactor at 160 °C for 3 min under 600 rpm stirring. The reaction mixture was then cooled in an ice bath for 2 min. The resulting liquid was separated immediately by filtration and diluted to 50 mL in a volumetric flask. The total phenolic content of this crude extract was measured (see Section 2.5.1). The solid algal residue was oven-dried at 60 °C for 48 h. Sub-samples of each species were extracted 3 times, each on a different day.

2.4. Characterisation of dried, milled algal powder seaweed and seaweed residue post-extraction

For all three species of seaweed and for each extraction protocol, the milled, dried seaweed powder and the algal residue post-extraction were analysed by scanning electron microscopy (SEM) to determine the effect of the extraction technique on cell morphology. Milled, dried seaweed powder and the dried algal residues were washed 3 times with 0.1 M phosphate buffer (pH 6.8). The buffer was removed by centrifugation at 4000 rpm for 5 min and the samples were fixed in a freshly prepared mixture of 2.5%v/v glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) at room temperature for 1 h. Subsequently, the samples were washed and dehydrated in a graded series of aqueous ethanol solutions (30–100%, v/v) and then dried using hexamethyldisilazane (HMDS). The samples were coated with a 15 nm thick gold film using an Emitech K550 sputter coater prior to being visualised and photographed with a scanning electron microscope (JEOL JCM-6000, NeoScope™).

2.5. Quantification of phlorotannins

2.5.1. Phlorotannins recovery

For all three species of seaweeds, phlorotannins were quantified using the Folin-Ciocalteu (F-C) method as described in Magnusson et al. [41]. For the RTE and CHE extraction methods, the values obtained in the current study were compared with the previously published values for the MAE and SLE methods [28].

2.5.2. Ferric-reducing antioxidant power (FRAP)

For all three species of seaweeds, FRAP of the crude extracts obtained by MAE was determined following the procedure of Chew et al. with minor modifications [42] as follows. A mixture of 0.1 M pH 6.6 potassium phosphate buffer (500 μL), 1% (w/v) potassium ferricyanide (500 μL), Milli-Q water (180 μL) and algal extract (180 μL) were incubated at 50 °C for 20 min. After cooling to room temperature, the solution was added to 10% w/v trichloroacetic acid (500 μL), Milli-Q water (1.7 mL) and 0.1%w/v aqueous iron(III) chloride (340 μL). The mixture was incubated at room temperature for an additional 30 min for colour development before the absorbance at 700 nm was measured on a UV–Vis spectrometer (Cary 60, Agilent Technologies, Australia). Calibration curves were obtained using 200 μL aliquots of aqueous gallic acid solutions (0, 5, 10, 20, 40, and 50 μg mL^{-1}) under the above FRAP assay conditions. The results of the FRAP assay are expressed as mg of gallic acid equivalents (GAE) per gram of algal dry weight.

2.5.3. DPPH radical scavenging activity

For all three species of seaweeds, DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity for the crude extracts obtained by MAE was determined following the procedure of Yan et al. with minor modifications [43]. Briefly, varying amounts of sample (2–200 μL diluted to 1 mL with water) and 2 mL of freshly prepared 0.15 mM DPPH in ethanol were mixed and sealed. After incubation at room temperature for 30 min in the dark, the absorbance at 520 nm was measured by UV–Vis spectrometer (Cary 60, Agilent Technologies, Australia). DPPH radical scavenging ability was calculated as:

$$\left(1 - \frac{A_{sample}}{A_{control}}\right) * 100$$

where $A_{control}$ is the absorbance of the mixture of 1 mL water and 2 mL ethanolic DPPH solution. The higher the DPPH radical scavenging ability, the higher the antioxidant activity. Calibration curves were obtained using 200 μL aliquots of aqueous gallic acid solutions (0, 5, 10, 20, 40, and 50 μg mL^{-1}) under the above DPPH assay conditions. Results are expressed as milligrams of GAE per gram of algal dry weight.

2.6. Thermal stability of phlorotannins

Thermal degradation of phlorotannins was determined following previous method [44] with minor modifications, by monitoring the decline in the ability to scavenge DPPH free radicals. DPPH radical scavenging activity was measured as described in Section 2.5.3 with 20 μL of ascorbic acid solution or freeze dried algal extract (see Section 2.3.1) solution amounting to 1 mL, in both the equal concentration assay (see Section 2.6.1) and equal initial activity assay (see Section 2.6.2). Results are expressed as % DPPH radical scavenging ability.

Table 1
Phlorotannin recovery (PGE % of dw) of *C. flexuosum*, *C. plumosum*, and *E. radiata*, using different extraction methods. (PGE: phloroglucinol equivalents; RTE: room temperature extraction; CHE: conventional hydrothermal extraction; SLE: conventional solid-liquid extraction; MAE: microwave assisted extraction.)

Extraction methods	Phlorotannin recovery (PGE % of dw)		
	C. flexuosum	*C. plumosum*	*E. radiata*
RTE	4.7 ± 0.4	5.4 ± 0.4	0.9 ± 0.0
CHE	8.8 ± 0.8	8.8 ± 0.7	1.6 ± 0.1
SLE [41]	8.6 ± 0.2	7.5 ± 0.1	1.5 ± 0.2
MAE [41]	15.8 ± 0.3	9.2 ± 0.6	2.0 ± 0.1

2.6.1. Thermal stability assay at equal initial concentration

For all 3 species of seaweeds, phlorotannins obtained by MAE were employed. Both the concentration of ascorbic acid and the concentration of algal extract were fixed at 0.75 mg/mL. The concentration of phlorotannin in the algal extracts was calculated from the results of the total phenolic content, measured by the F-C assay (Section 2.5.1). Samples were incubated at 30 °C and 60 °C in the dark for 7 days and tested daily, where day 0 refers to freshly prepared solutions from freeze-dried extracts.

2.6.2. Thermal stability assay with comparable initial activity

For *C. flexuosum* and *C. plumosum* phlorotannins obtained by MAE were employed. Solutions of different concentrations were prepared in order that all the samples displayed similar radical scavenging abilities initially. Based on the results of the F-C assays for total phenolic content, the initial concentrations of the solutions were: 0.75 mg/mL ascorbic acid; 1.29 mg/mL PGE (phloroglucinol equivalents) for *C. flexuosum*; 1.69 mg/mL PGE for *C. plumosum*. Samples were incubated at 30 °C and 60 °C in the dark for 7 days and tested daily, where day 0 refers to freshly prepared solutions from freeze-dried extracts. The thermal stability of *E. radiata*-derived phlorotannins was not included because the concentration of phlorotannins in the raw extract was so low it was not possible to prepare a solution with DPPH radical scavenging ability comparable to that of other crude algal extracts, or ascorbic acid.

2.7. Statistical analysis

The effects of the extraction method (MAE, SLE, RTE, CHE) on the yield of phlorotannins from each of the three species of seaweed was evaluated using two-factor ANOVA, with the extraction method and species as the factors. Yield data were transformed (log + 1) to improve homogeneity of variances prior to analysis. Where ANOVA resulted in a significant difference in means ($\alpha = 0.05$), Tukey's multiple comparison tests were used to compare the means of treatment groups. The variance component (% variance explained, η^2) was calculated to interpret the relative importance of the significant terms in the model as there were interactions between the two factors. Differences in antioxidant capacity (FRAP and DPPH) between extracts from the three species were compared using one-way ANOVAs. All analyses were performed in Statistica v12, StatSoft Inc.

2.8. Purification of *C. flexuosum* phlorotannins by liquid–liquid extraction (LLE)

Liquid–liquid partition with ethyl acetate is a standard technique for the isolation of phlorotannins from crude aqueous extracts of seaweed [45,46]. Here, 500 mg of freeze-dried crude algal extract (from MAE or conventional SLE) was dissolved in 25 mL Milli-Q water. The algal extract solution was partitioned with 2 × 25 mL dichloromethane (DCM) to remove lipids. The resultant aqueous phase was extracted with 3 × 25 mL ethyl acetate and the ethyl acetate extract was then concentrated at 25 °C under reduced pressure and dried under vacuum for 24 h, affording 13.9–34.0 mg of purified phlorotannins. The remaining aqueous fraction was freeze dried. The isolated phlorotannins were either dissolved in D_2O for NMR analysis, or diluted to 1 mg/mL in a mixture of HPLC grade acetonitrile and water (1:1) for HPLC-MS analysis. ^{1}H NMR 300 MHz spectra were measured on a Bruker Ultrashield™ 300 spectrometer at 298 K in D_2O.

2.9. Characterisation of phlorotannins

HPLC-DAD-ESI-MS analysis was used to characterise the phlorotannins extracted from *C. flexuosum*. Chromatographic analyses were carried out using a Shimadzu LCMS-2010EV equipped with an autosampler, two binary pumps, a column oven, and a DAD detector, over a Phenomenex C-18 bonded silica column (P/NO: 00G-4252-E0, 250 × 4.6 mm, 5-micron particle size). Acetonitrile and water containing 0.1%v/v formic acid were used as eluents, gradient elution: acetonitrile 0–60%v/v over 60 min. The flow rate was 0.8 mL/min at 40 °C and the UV–vis spectra recorded at 280 nm. Mass spectra were monitored in positive ion mode, scanning from m/z 100 to m/z 2000. The detector voltage was 1.5 eV and the drying gas (N_2) temperature was 300 °C. The nebulizer gas was generated by a domnick hunter® NITROX® Nitrogen Generator, flow rate 1.5 L/min.

3. Results and discussion

3.1. A comparison of the efficiency of extraction methods

Three species of brown seaweeds (*C. flexuosum*, *C. plumosum*, *E. radiata*) were extracted by using 4 protocols: MAE, SLE, CHE and RTE. The extraction efficiency in terms of phlorotannin recovery and the changes to cellular structure due to extraction were compared (Table 1 & Fig. 2). Both species of seaweed and extraction method significantly affected the extraction yield of phlorotannins, with an interaction between the two factors (ANOVA, $F_{6,24} = 12.82$, $p < 0.001$, Supporting information, Table S2). Notably, 84.0% of the variance was explained by species (Table S2), with a 41.8%–87.3% higher yield for *C. flexuosum* extracted by MAE compared with *C. plumosum* and *E. radiata* (Table 1), while 13.1% of the variance was explained by the extraction method used, with a 44.3%–70.3% higher yield for *C. flexuosum* extracted by MAE compared with RTE, SLE, and CHE (4.3%–41.3% for *C. plumosum* and 20%–55% for *E. radiata*). *Ecklonia radiata* consistently had the lowest yield of phlorotannins. Importantly, all species showed the same trend in extraction efficiency across the four assessed methods: MAE > CHE > SLE > RTE.

3.2. Characterisation of dried, milled algal powder before, and solid algal residue after, extraction

In order to understand the increased efficiency of MAE for phlorotannin recovery, algal cell morphologies were investigated by examining solid algal samples by SEM to assess the degree of damage to the cell wall before and after extraction. For *C. flexuosum*, the dried, milled algal power before extraction (shown in Fig. 2A1) consists of more or less intact cell walls, while the cells in the extracted algal residues exhibit varying degrees of destruction (Fig. 2A2–A5).

Stirring at room temperature for 10 min in water (RTE) (Fig. 2A2) was not sufficient to destroy the algal cell wall, however, minor distortion of the cells and the removal of fine particulate material around the cell wall and inside the cell was observed. With increased extraction time under harsher conditions, significant changes to the cell wall integrity occurred. Sequential 24 h SLE with aqueous, acidified methanol (50%v/v, pH 2), followed by aqueous acetone (70%v/v, pH 2), with a biomass: solvent ratio of 1:40 (SLE), resulted in only a few of the cells remaining intact (Fig. 2A3). Under microwave-assisted extraction (MAE) conditions (160 °C in water; autogenous pressure) an even

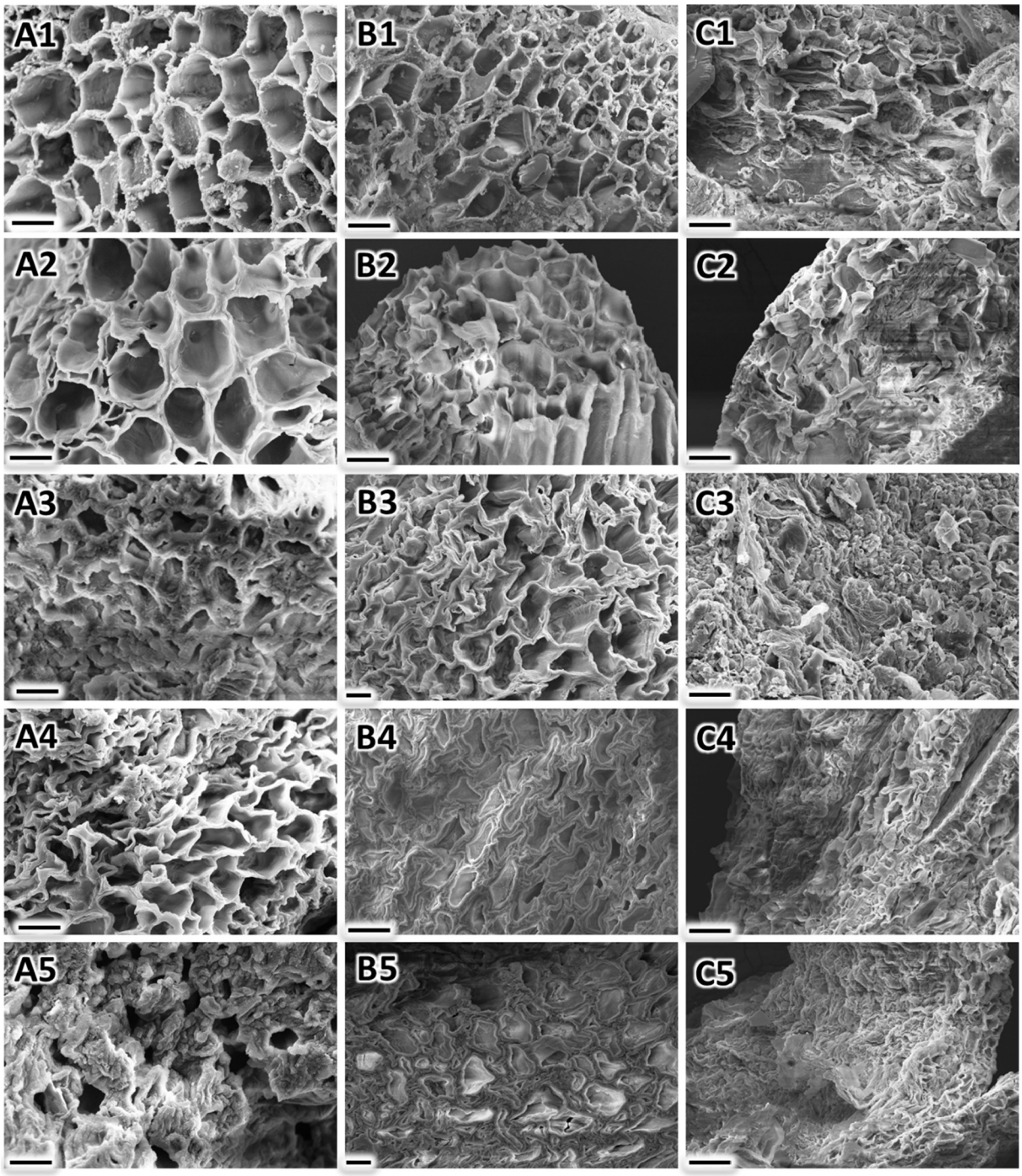

Fig. 2. Scanning electron microscopy (SEM) images of dried, milled brown seaweed before and after extraction. Different letters demonstrate different species of seaweeds (A) *C. flexuosum*; (B) *C. plumosum*; (C) *E. radiata*. Different numbers demonstrate different kinds of solids (1) Before extraction; (2) algal residue after RTE (room temperature extraction); (3) algal residue after SLE (conventional solid-liquid extraction); (4) algal residue after CHE (conventional hydrothermal extraction); (5) algal residue after MAE (microwave assisted extraction). Magnification, 700-fold. The scale bars are: 20 μm for all except B3 & B5 (10 μm).

greater degree of cell wall degradation took place. A pronounced lack of cellular structure was observed by SEM (Fig. 2A5). Although it is not possible to conclude from these micrographs whether complete removal of phlorotannins from the seaweed had taken place, a clear association between the recovery of phlorotannins and the extent of cell wall degradation was observed.

To better compare microwave heating and conventional heating, including the influence of pressure, an autoclave in an oil bath (CHE) was used for the extraction of phlorotannins and the residual seaweed was also imaged by SEM (Fig. 2A4). It is clear that the degree of cell wall distortion is greater than that resulting from room temperature extraction (*cf* Fig. 2A4 and A2) but less than that resulting from MAE (*cf* Fig. 2A4 and A5).

For *C. plumosum*, the dried, milled algal powder before extraction also exhibited the more or less intact cells (Fig. 2B1). RTE of *C. plumosum* also resulted in slight distortion of the cells (Fig. 2B2). Clearly, the cell wall distortion from SLE is greater than RTE (*cf* Fig. 2B3 and B2) but weaker than CHE and MAE (*cf* Fig. 2B3 and B4, B5). Similar to *C. flexuosum*, there was a clear association between the recovery of phlorotannins and the extent of cell wall degradation.

Compared with the two *Carpophyllum* species, interpretation of the SEM data for *E. radiata* was not straightforward. It was not obvious that the cells were intact in the powdered algal sample before extraction (Fig. 2C1). Additionally, the apparent low phlorotannin content of *E. radiata* makes it difficult to confidently associate the recovery of phlorotannins with the degree of distortion of the cell wall, with no major differences in extraction efficiency being observed for method employed.

Phlorotannins in brown seaweed are present in two main locations within the cell [47]. The soluble phlorotannins reside in the physodes. These are cytoplasmic organelles present in brown seaweed or other seaweed that are typically spherical to elliptical, highly mobile and refract light strongly [48,49]. Cell wall bound phlorotannins are insoluble due to their interaction with the polysaccharides in forming, *e.g.*, alginate-phlorotannin complexes [50]. The extraction conditions for soluble phlorotannins are usually mild (organic solvents at room temperature), while the extraction of cell-wall-bound phlorotannins requires much harsher conditions, *e.g.*, 1 M sodium hydroxide at 80 °C for 2.5 h, to break up the cell wall [49].

Although it is not possible to determine if the cell-wall-bound phlorotannins were extractable on the basis of the cell morphology alone, clearly MAE resulted in the greatest degree of cell wall distortion and gave the highest recovery of phlorotannins for the 3 species of brown seaweed (Table 1 & Fig. 2). Therefore, one of the possible reasons that MAE facilitates the highest recovery is that the microwave conditions were sufficiently energetic to partially depolymerise the algal cell wall resulting in the release of cell wall-bound phlorotannins.

3.3. Antioxidant activity of the extracted phlorotannins

As MAE was the best extraction method among the 4 methods studied, the antioxidant activities of MAE extracted phlorotannins from all three species of brown seaweeds were assessed by FRAP activity and DPPH radical scavenging ability, and then compared with the total phenolics content [41]. Significant differences in antioxidant activities were observed among the three species of brown seaweed (FRAP ANOVA, $F_{2,6} = 289.2$, $p < 0.05$, DPPH ANOVA, $F_{2,6} = 660.7$, $p < 0.05$, Supporting information, Table S3). *Carpophyllum flexuosum* showed the highest FRAP activity (62.1 ± 4.8 mg GAE/g dw), followed by *C. plumosum* (33.0 ± 1.8 mg GAE/g dw), with *E. radiata* having the lowest FRAP activity (4.4 ± 0.4 mg GAE/g dw) (Fig. 3). A similar trend occurred for DPPH radical scavenging ability, where *C. flexuosum* had the highest activity (37.4 ± 2.1 mg GAE/g dw), followed by *C. plumosum* (11.9 ± 0.2 mg GAE/g dw) and *E. radiata* (2.5 ± 0.22 mg GAE/g dw) (Fig. 3). When ascorbic acid was subjected to the FRAP assay its activity was 369.4 ± 38.8 mg GAE/g, showing that 6 g of dried *C. flexuosum* biomass equivalent to 3 g of extracts on a dry basis has the equivalent FRAP activity of 1 g of pure ascorbic acid.

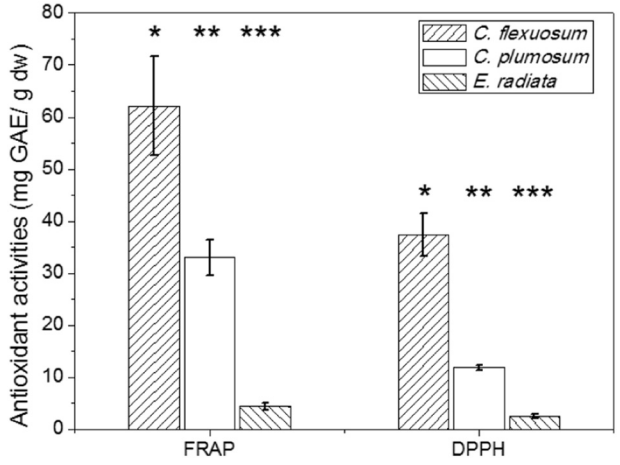

Fig. 3. The antioxidant activities of extracts from seaweed *C. flexuosum*, *C. plumosum* and *E. radiata* obtained by MAE, determined by ferric-reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability. Results are expressed as gallic acid equivalents (GAE) per gram of algal dry weight as mean ± 2 stdev, n = 3. A different number of asterisks (*) indicates significant differences between species within assays (FRAP or DPPH). Details of statistical analysis are shown in the Supporting information, Table S3.

When comparing with TPC [41], a strong association or relationship was observed between the antioxidant activities (FRAP and DPPH) and TPC (Fig. 4A & B). Samples with higher content of phlorotannins had greater antioxidant capability suggesting that phlorotannins are the major class of antioxidants within the extract. This is in accordance with previous research, where a strong antioxidant activity has been related to a high content of polyphenols [21,51,52].

3.4. Thermal stability of phlorotannins extracted from brown seaweed

The thermal stability of phlorotannins extracted from *C. flexuosum*, *C. plumosum* and *E. radiata* by MAE was quantified by monitoring the comparative decline of DPPH radical scavenging ability of aqueous solutions, at elevated temperature with the common antioxidant ascorbic acid. The thermal stability was compared at both equal concentration and equal initial radical scavenging ability.

Setting the initial concentrations of phlorotannins in the extracts and ascorbic acid at 0.75 mg/mL (see Section 2.6.1), allowed for a comparison of the initial radical scavenging ability and thermal stability over 7 days at 30 °C (Fig. 5A) and 60 °C (Fig. 5B). Under these conditions, ascorbic acid initially scavenged the highest amount of DPPH free radicals (66.2%), followed by the extract of *C. flexuosum* (56.0%) and *C. plumosum* (25.3%), while the extract of *E. radiata* scavenged the lowest amount (2.84%) (Fig. 5A & B). Despite these initial differences in activity, all the algal extracts were more stable than ascorbic acid at both assay temperatures. At 30 °C (Fig. 5A), by day 4, the DPPH scavenging ability of *C. flexuosum*-derived phlorotannins was more than twice that of ascorbic acid. By day 7, the DPPH scavenging ability of *C. flexuosum*-derived phlorotannins was > 10-fold that of ascorbic acid. The DPPH scavenging ability of *C. plumosum*- and *E. radiata*-derived extracts were stable at 30 °C for 7 days. The changes in DPPH scavenging ability at 60 °C (Fig. 5B) showed an even more pronounced decline for ascorbic acid. By day 2, the DPPH scavenging ability of *C. flexuosum*-derived phlorotannins was nearly 4-fold that of ascorbic acid, and > 14-fold after 7 days incubation at 60 °C.

For the second assay, the use of crude brown algal extracts as natural antioxidants to replace ascorbic acid was investigated. Concentrations of the crude extracts of *C. flexuosum* and *C. plumosum* were prepared such that their initial radical scavenging abilities were comparable to a 0.75 mg/mL solution of ascorbic acid. The initial DPPH scavenging ability for the solutions thus prepared was all within 60–70% (Fig. 6). At both temperatures, the algal extracts retained a higher degree of activity than ascorbic acid over the 7-day testing period. Additionally, after day 2, the activities of the algal extracts were similar regardless of the incubation temperature. A possible reason for this phenomenon is that the crude extract contains a mixture of reactive phlorotannins, which could be easily oxidised, in addition to there being more robust, stable phlorotannins.

Overall, our results clearly demonstrate that the algal phlorotannins are much more thermally stable than ascorbic acid and that phlorotannins extracted from *C. flexuosum* and *C. plumosum* can have a wide variety of applications as natural antioxidant materials. Kang et al. reported that dieckol, a phlorotannin extracted and isolated from brown seaweed *Ecklonia cava*, had similar initial radical scavenging ability as ascorbic acid but much greater stability at 90 °C for up to 7 days [44]. This is the first time, however, that crude algal extracts have been studied by means of a thermal stability test for their suitability to replace ascorbic acid as a natural antioxidant. The obvious benefit of using whole extracts as natural antioxidant is that they are

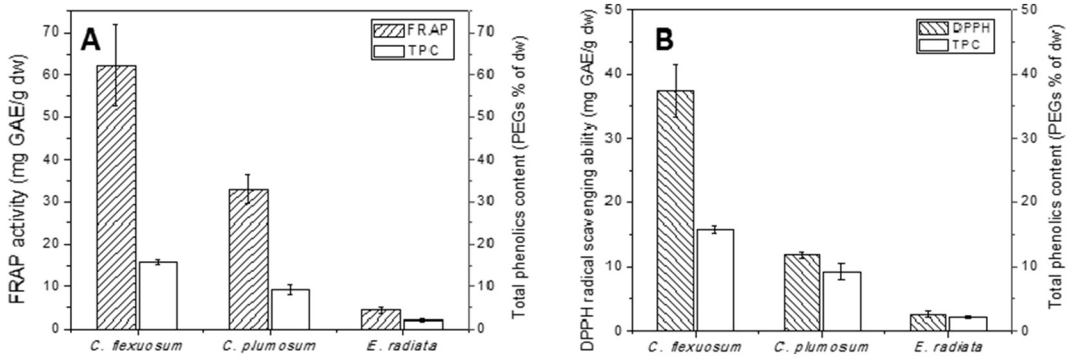

Fig. 4. Relationship between the antioxidant activity of the algal extracts from MAE and total phenolic content (TPC). (A) Ferric-reducing antioxidant power (FRAP) activity (B) 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity compared with TPC from *C. flexuosum*, *C. plumosum* and *E. radiata*. Results are expressed as gallic acid equivalents (GAE) per gram of algal dry weight. Data is presented as mean ± 2 stdev, n = 3.

easy to prepare and purification can be avoided. These results show that *C. flexuosum* phlorotannins may be of significant therapeutic value due to their inherently high antioxidant activity and thermal stability.

3.5. Purification of phlorotannins

As *C. flexuosum* had the highest phlorotannin content (Table 1), the highest antioxidant activity per equivalent unit of phlorotannins (Fig. 5), a high radical scavenging activity after 7 days (Figs. 5 & 6), and the largest difference in yield of phlorotannins between extraction methods (SLE and MAE) (Table 1), phlorotannins from *C. flexuosum* extracted by MAE and SLE were selected for further purification and characterisation to identify their molecular structures.

^{1}H NMR analysis was used to gain information about the components present in the initial crude extract, *i.e.*, the phlorotannins extracted into ethyl acetate, as well as those remaining in the aqueous fraction. NMR spectra (Figs. 7 & 8) showed that the composition of the crude extract from *C. flexuosum* resulting from MAE and SLE were similar. The major products were identified as phlorotannins (δ = 6.5–5.5) (Fig. 7, *middle panel* & Fig. 8, *middle panel*) and polysaccharides (δ = 4.0–3.5) (Fig. 7, *top panel* & Fig. 4, *top panel*). After first removing non-polar products by extracting the crude extract with dichloromethane, the NMR analysis confirmed that the majority of the phlorotannins were separated from the aqueous polysaccharides by partitioning them into ethyl acetate (Figs. 7 & 8, *middle and bottom panels*).

Despite the similarities in the appearance of the ^{1}H NMR spectra for the crude algal extracts obtained by means of MAE and SLE, some slight differences were apparent. For example, there were a greater number of absorptions assigned to the polysaccharides in the MAE extract (Fig. 7, *top & bottom panels*) (δ = 4.1–3.9) than in the corresponding region of the spectrum of the SLE extract in Fig. 8, *top & bottom panels*. This difference was probably due to a greater number of polysaccharide-derived species obtained by MAE: the more efficient of the two extraction protocols. Also, the shape and relative intensity of the phlorotannin absorptions (δ = 6.5–5.5) are different between the algal extracts obtained from MAE (Fig. 7, *middle panel*) and SLE (Fig. 8, *middle panel*). One possible reason for this is that some of the phlorotannin species oxidatively degraded or reacted during the extraction process. Alternatively, structurally different phlorotannin species were obtained by the two extraction methods. Further structural details of the extracted phlorotannins were obtained using HPLC-MS (Section 3.6).

3.6. Characterisation of phlorotannins extracted from C. flexuosum

HPLC-MS analysis was performed to characterise the major phlorotannins present in the ethyl acetate fractionated extracts of *C. flexuosum* from MAE and conventional SLE. In terms of the phlorotannins extracted by MAE, the baseline of the diode array detector (DAD) chromatogram was flat and 15 peaks were well separated within a 25-minute window (Fig. 9, A). Moreover, the signal pattern of the total ion current (TIC) chromatogram (Fig. 9, B) corresponded closely to that of the DAD chromatogram. However, for those phlorotannins extracted by conventional SLE (Fig. 10), the baseline of the DAD chromatogram was not flat (Fig. 10, A) and there was an elevated baseline between 16 min and 45 min (Fig. S1). In addition, within the initial 25-minute window, more peaks were observed in the total ion current (TIC) chromatogram (peaks 7–14) than in the DAD chromatogram (Fig. 10, B). On the basis

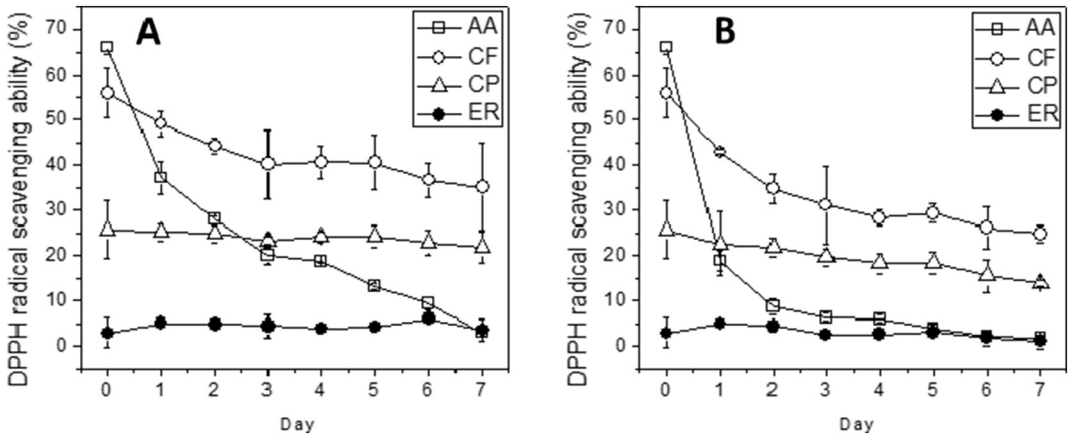

Fig. 5. Reduction in 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of phlorotannins extracted from *C. flexuosum* (CF), *C. plumosum* (CP) and *E. radiata* (ER) and compared with ascorbic acid (AA). Samples were incubated at 30 °C (A) and 60 °C (B). Initial sample concentration 0.75 mg/mL for all samples. Data is presented as mean ± 2 stdev, n = 3.

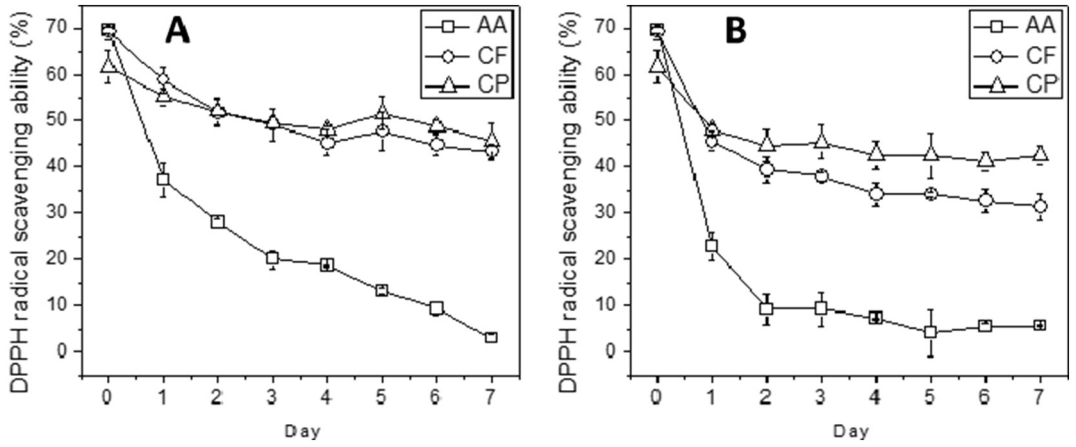

Fig. 6. Reduction in 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of phlorotannins extracted from *C. flexuosum* (CF) and *C. plumosum* (CP) compared with ascorbic acid (AA). Samples were incubated at 30 °C (A) and 60 °C (B). The concentrations of the solutions were adjusted to afford comparable initial activity. Data is presented as mean ± 2 stdev, n = 3.

of analysis of their parent- and fragment-ion *m/z* values, these peaks were not able to be assigned to any obvious phlorotannin species. By comparing the number of peaks in the DAD chromatograms (Figs. 9A & 10A), six major chemical species were detected within the phlorotannins extracted by MAE. There were only four major species detected for the phlorotannins extracted by conventional SLE. Possibilities for this difference are that MAE is more efficient at breaking the algal cell wall than SLE is, resulting in a greater number of species being extracted from the cell wall, or that the rapid release of the phlorotannins from brown seaweed (3 min *vs* 48 h) prevented the phlorotannins from oxidation. The presence of numerous oxidation products in the conventional SLE extract could account for the broad baseline observed. Therefore, MAE is a much milder method, yielding less degraded extracts.

By analysing the mass spectrum of each peak (Tables 2 & 3; peak numbers correspond to those in Figs. 9 & 10) and comparing with literature data [53–55], several phlorotannin compounds were identified.

For example, in Fig. 9: peaks 8, 9, and 10 each give rise to a mass spectral peak at *m/z* 795. This was assigned to the protonated molecular ion ($[M + H]^+$) of an isomer of a bifuhalol trimer (Table 2). The product ions at *m/z* 655 (loss of 140); 515 (further loss of 140) and 391 (further loss of 124) can be rationalised on the basis of sequential cleavage of arylether bonds, with concomitant neutralisation of the intermediate radical by hydrogen. Similarly, compounds 12 and 13 with their assigned protonated molecular ions at *m/z* 779 were assigned as the isomers of hydroxyhexafuhalol (Table 2). The above two examples of species assignment are further illustrated in Fig. 11. Putative structures of an isomer of bifuhalol and hydroxyhexafuhalol are shown along with their proposed, respective ion fragmentations. Further structural assignments and fragmentation patterns are given in the Fig. S2. In addition, some evidence for oxidative changes under mass spectral conditions were observed in peaks 12 (*m/z* 513) and 14 (*m/z* 653) in Fig. 9. See the Supporting information, Fig. S2 for details.

On the basis of the above analysis, the major phlorotannins in *C. flexuosum* belong to the fuhalol group (Fig. 1). In general, the separation and analysis of phlorotannins by HPLC-MS is complicated due to the large number of compounds in the extract having similar structures and physical properties. In this study, this difficulty is exacerbated by the additional complexity introduced due to the presence fuhalols and hydroxyfuhalols: existing as several isomers with at least 1 extra

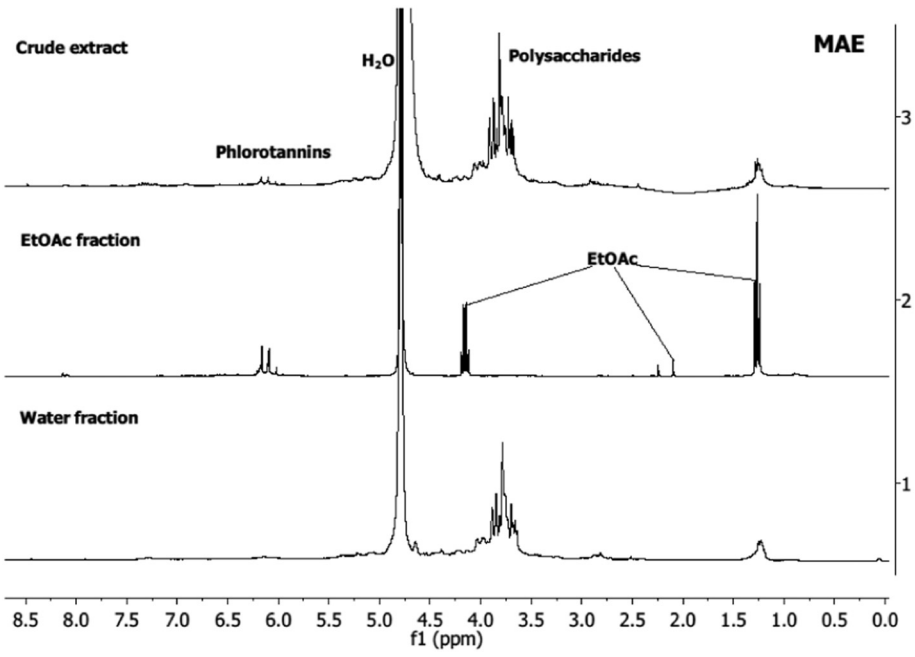

Fig. 7. ^{1}H NMR spectra of the crude aqueous extract of *C. flexuosum* obtained from microwave assisted extraction (MAE), ethyl acetate fraction and remaining water fraction.

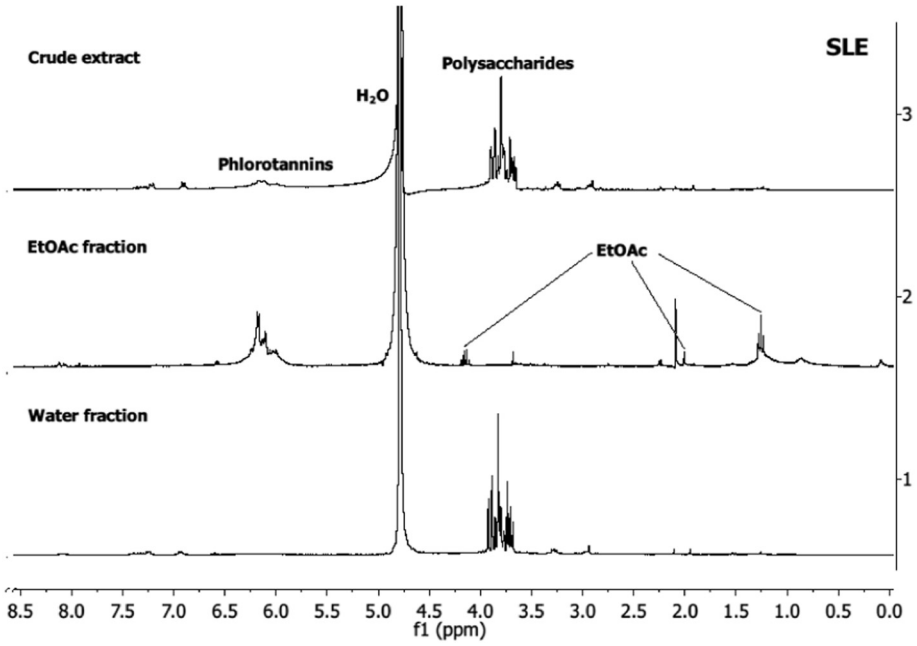

Fig. 8. ^{1}H NMR spectra of the crude aqueous extract of *C. flexuosum* obtained from conventional solid-liquid extraction (SLE), ethyl acetate fraction and remaining water fraction.

hydroxyl group substituent [56–58]. These difficulties notwithstanding, several groups have made significant progress in identifying fuhalols from other species of brown seaweed. For example, Glombitza and Schmidt identified 45 phlorotannins from *Carpophyllum angustifolium*, of which 37 are of the fuhalol structural type [59]. Li et al. also identified 20 fuhalols and 15 eckols among 42 phlorotannins from *Sargassum fusiforme* [60].

To the best of our knowledge, this is the first time the characterisation of the phlorotannins in *C. flexuosum* has been reported. It is worth noting that the actual mass of the phlorotannins isolated by ethyl acetate extraction of the crude aqueous extract was much lower than what was indicated by the F-C assay (MAE: 6.8% dw *vs* 15.8% PGE of dw & SLE: 2.8% dw *vs* 8.6% PGE of dw). This is clearly due to the differences in the inherent antioxidant capacities of pure phloroglucinol and the structurally diverse mixture of extracted phlorotannins. For example, Thavasi et al. reported that the *ortho*-arrangement of OH-groups in trihydroxy- and dihydroxy-benzenes allows for intramolecular hydrogen bonding between adjacent OH-groups, which increased the rate and ability of DPPH radical scavenging [61]. Fuhalols, which were identified as the major components of *C. flexuosum* phlorotannins, also contain this structural motif and hence are expected to have higher activity than the equivalent mass of phloroglucinol.

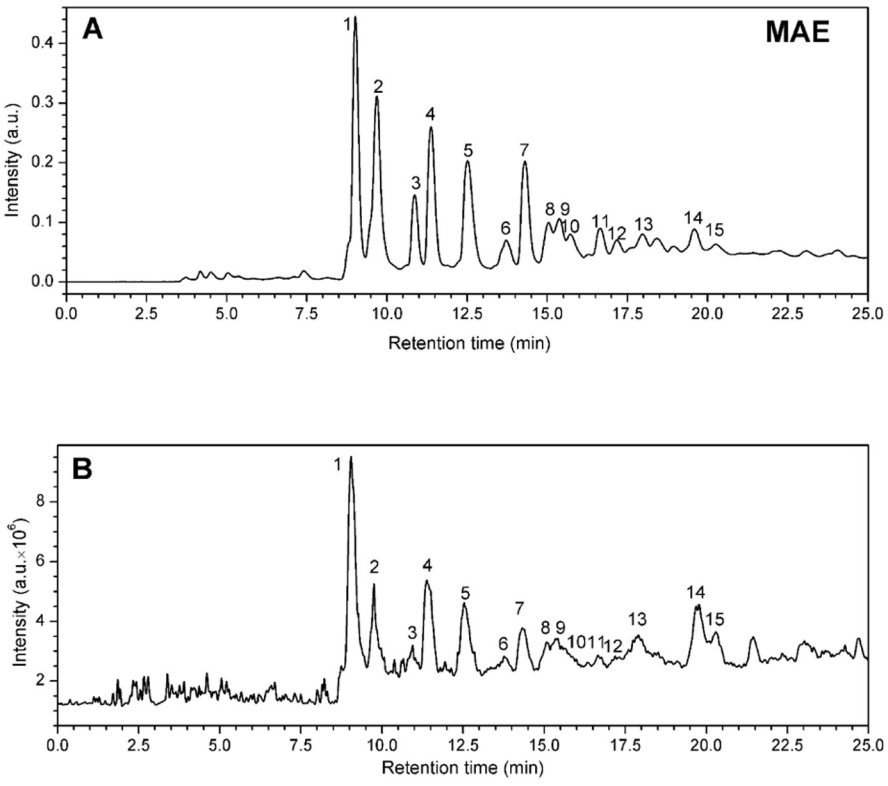

Fig. 9. High-performance liquid chromatography with a diode-array detector (HPLC-DAD) chromatogram (A) and total ion current (TIC) chromatogram (B) of the fractionated phlorotannin extracts of *C. flexuosum* by microwave assisted extraction (MAE), recorded at 280 nm.

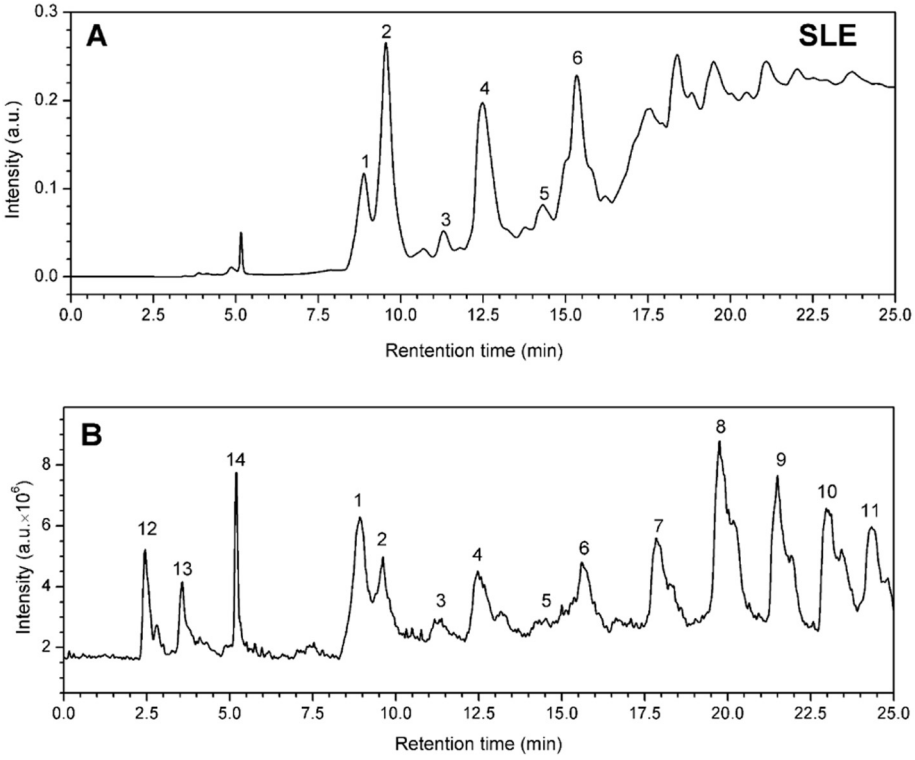

Fig. 10. High-performance liquid chromatography with a diode-array detector (HPLC-DAD) chromatogram (A) and total ion current (TIC) chromatogram (B) of the fractionated phlorotannin extracts of *C. flexuosum* by conventional solid-liquid extraction (SLE), recorded at 280 nm.

The post-extraction cell morphology, observed by SEM (Fig. 2), in conjunction with the comparison of the HPLC-MS data of phlorotannins extracted by conventional SLE and MAE supported the hypothesis that microwave heating was able to degrade the algal cell wall efficiently, leading to the release of phlorotannins before they were oxidised. For example, peak 4 in Fig. 9 and peak 3 in Fig. 10, have similar retention times (11.37 and 11.38 min respectively) but distinct and different mass spectral fragmentation patterns. Peak 4 in Fig. 9 is probably a bifuhalol isomer (Table 2), whereas peak 3 in Fig. 10 did not correspond to an obvious phlorotannin structure. Further study of the mass spectral pattern of peak 3 in Fig. 10, however, led to its assignment as an oxidised phlorotannin species: shown in Fig. 12, in which a possible isomer of this species and the hypothesised fragmentation pathway are illustrated.

Table 3
Retention times and mass spectrometric data of phlorotannins in *C. flexuosum*, isolated by conventional solid-liquid extraction (SLE), determined by HPLC-ESI-MS. Peak numbers correspond to those in Fig. 10.

Peak number	Suggested phlorotannin	Retention time (min)	MW	Parent ion ([M + H]$^+$) & product ion *m/z*
1	Bifuhalol	8.89	266	267
2	Hydroxytrifuhalol	9.55	406	407, 268, 154
3	Unassigned	11.38	–	653, 391, 251, 139
4	Bifuhalol dimer	12.47	530	531, 391
5	Trifuhalol	14.35	390	391
6	Bifuhalol trimer	15.37	794	795, 655, 515, 391, 261, 130

Table 2
Retention times and mass spectrometric data of microwave assisted extraction (MAE) extracted phlorotannins in *C. flexuosum* determined by HPLC-ESI-MS. Peak numbers correspond to those in Fig. 9.

Peak number	Suggested[a] phlorotannin	Retention time (min)	MW	Parent ion ([M + H]$^+$) & product ion *m/z*
1	Bifuhalol	9.01	266	267, 127
2	Hydroxytrifuhalol	9.68	406	407, 391, 361, 267, 249, 226, 154, 129
3	Unassigned	10.86	–	1200, 842, 554, 367, 253, 198
4	Bifuhalol	11.37	266	267, 245
5	Bifuhalol dimer	12.53	530	531, 391
6	Bifuhalol dimer	13.77	530	531
7	Trifuhalol	14.29	390	391, 130
8	Bifuhalol trimer	15.01	794	795, 655, 515, 391, 300, 227
9	Bifuhalol trimer	15.37	794	795, 655, 515, 391, 300, 245, 227
10	Bifuhalol trimer	15.72	794	795, 655, 515, 391, 300, 261, 227
11	Tetrafuhalol	16.67	514	515
12	Hydroxyhexafuhalol	17.18	778	779, 639, 513, 463, 391, 345, 261, 227
13	Hydroxyhexafuhalol	17.96	778	779, 639, 515, 470, 305, 243, 164, 129
14	Hydroxyoctafuhalol	19.60	1042	1043, 903, 779, 653, 529, 389, 372, 349, 221, 180
15	Unassigned	20.03	–	471, 393, 371, 238, 130

[a] Peak assigned to the same phlorotannin understood to be isomers of the suggested structure.

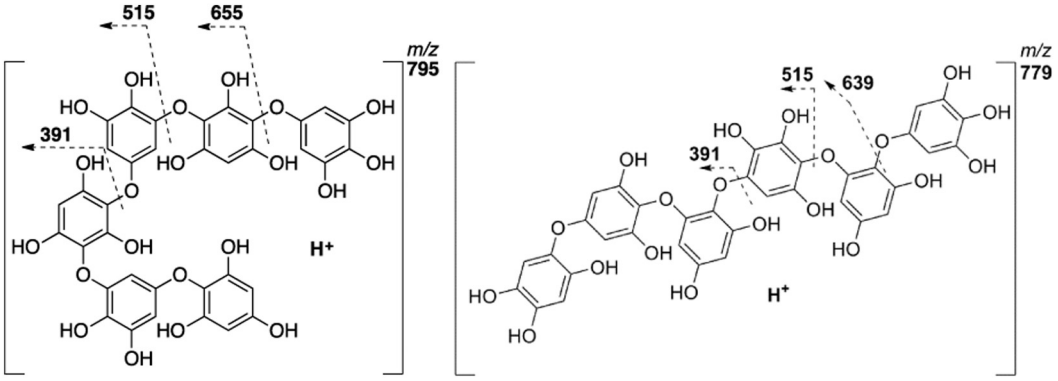

Fig. 11. Proposed fragmentation pathway of trimer of bifuhalol (MW 794) and hydroxyhexafuhalol (MW 778).

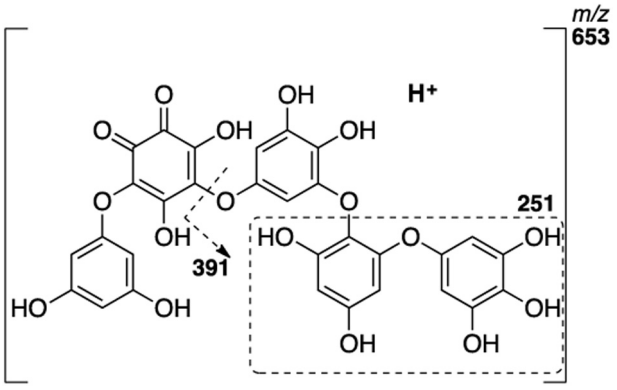

Fig. 12. Putative structure and fragmentation pathway for an oxidised product of phlorotannin peak 3, Table 3 (assuming MW 652).

4. Conclusions

The results from this study demonstrate that MAE was the most efficacious of the four methods used to recover phlorotannins from brown seaweeds, with *C. flexuosum* being the species chosen to showcase MAE technology. Efficient destruction of the cell wall of *C. flexuosum* by MAE led to the fast release of phlorotannins before they were degraded and oxidised to less biologically active materials. In the screening assays employed here, the phlorotannins extracted from *C. flexuosum* have potential to be used as natural antioxidant materials in many areas, having demonstrated antioxidant ability and a high degree of thermal stability in solution. The thermal stability of phlorotannins extracted from *C. flexuosum* is better than ascorbic acid by 5.5-fold after incubation at 60 °C for 7 days. The majority of the phlorotannins in *C. flexuosum* belong to the fuhalol class. Future studies will focus on the relationship between the molecular structure and biological activity of this and other classes of phlorotannins from *C. flexuosum* and the selective extraction of phlorotannins and polysaccharides using a biorefinery model.

Author's contribution

R. Zhang performed the research. R. Zhang wrote the manuscript with help from all co-authors. All authors were involved in the conception and design of the study, in analysis and interpretation of data and in critical revision of the manuscript. All authors have approved to submit this manuscript to *Algal Research*.

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Conflict of interest statement

No conflicts, informed consent, human or animal rights applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2017.11.027.

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