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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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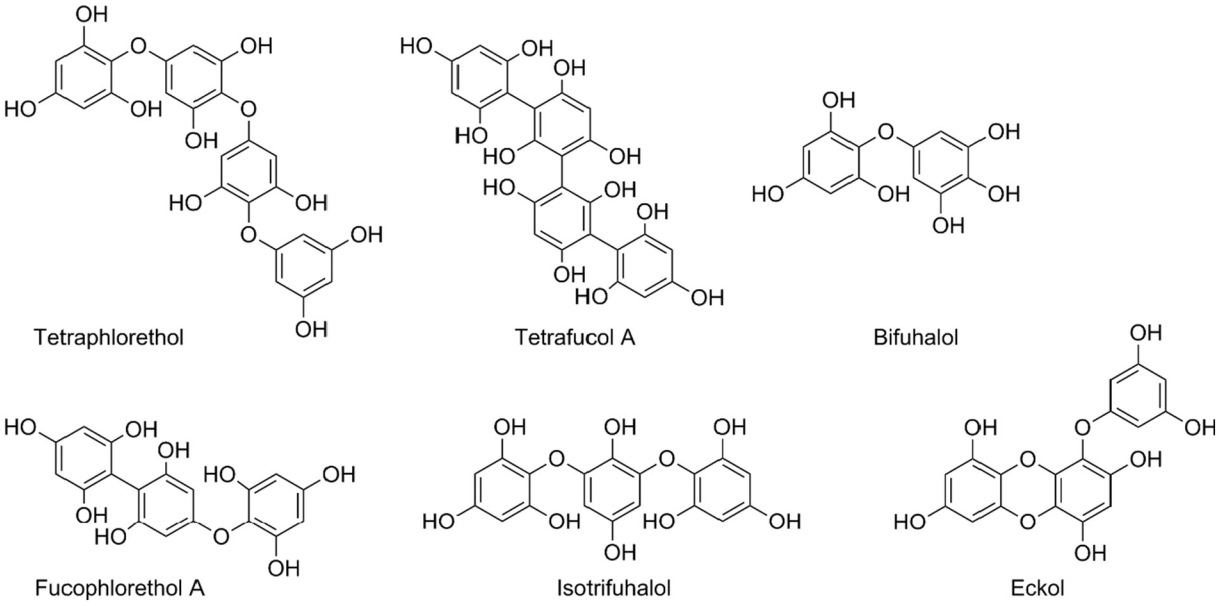

Fig. 1. Example structures of the 6 main classes of phlorotannins.

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