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Anti-proliferative and potential anti-diabetic effects of phenolic-rich extracts from edible marine algae

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ABSTRACT

Phenolic-rich extracts from four edible marine macroalgae commonly found in UK waters were tested for their potential biological effects towards cultured colon cancer cells and for their ability to inhibit digestive enzymes to achieve potential anti-diabetic effects. Extracts from *Palmaria, Ascophyllum* and *Alaria*, but not *Ulva*, gave reasonable recoveries of phenolics and inhibited the proliferation of colon cancer cells in a dose-responsive manner. *Alaria* extracts were more effective than *Palmaria* or *Ascophyllum* extracts, but *Palmaria* and *Ascophyllum* would provide greater amounts of phenolics per gram intake.

Extracts from *Palmaria, Ascophyllum* and *Alaria* all inhibited α-amylase activity to some extent, but *Ascophyllum* extracts were very effective with an IC_{50} of ~0.1 μg/ml GAE. The *Ascophyllum* extracts also inhibited α-glucosidase, the other key enzyme involved in starch digestion and blood glucose regulation, at low levels (e.g. IC_{50} ~20 μg/ml GAE).

After fractionation on Sephadex LH-20, the inhibitory activity from *Ascophyllum* was concentrated in the fraction which, from mass spectrometric evidence, was enriched in phlorotannins. These components have the capacity to inhibit α-amylase and α-glucosidase activities at μM levels, which are easily achievable in the gut. This may explain the anti-diabetic properties associated with algal extracts and algal phenolics in various *in vivo* studies.

1. Introduction

Edible marine macroalgae or seaweeds have formed an important part of the diet of many Far Eastern countries for centuries (Chan, Ho, & Phang, 2006) and although their use as foods in Western countries is well documented, they are less used. These marine macroalgae have been classified based on pigmentation into brown (Phaeophyta), red (Rhodophyta) and green (Chlorophyta) types. Seaweeds are greatly used in Eastern cuisine (Yuan, Carrington, & Walsh, 2005) and demand for ingredients, such as "Kombu" from *Laminaria* species and "Nori" used in sushi from *Porphyra* species, has been largely met from cultivation of the seaweeds. Apart from food uses, including their main industrial use as thickeners and gelling agents, seaweeds are widely used as ingredients in cosmetics and as fertilisers (McHugh, 2003). Seaweeds are ecologically important in the food chain as primary producers and some have demonstrated potential as chelators of heavy metals (Chan et al., 2006).

Edible seaweeds contain a range of components which have potential health benefits (MacArtain, Gill, Brooks, Campbell, & Rowland, 2007; Smit, 2004). They are good sources of dietary fibre, especially soluble fibre such as alginates, which can influence satiety and glucose uptake from foods (Brownlee et al., 2005). These soluble polysaccharides may also act as prebiotics, stimulating growth of "beneficial" bacteria in the colon (Wang, Han, Hu, Li, & Yu, 2006). As well as being sources of polyunsaturated fatty acids, minerals and certain vitamins (MacArtain et al., 2007), edible seaweeds can contain appreciable amounts of polyphenols (Rodriguez-Bernaldo de Quiros, Lage-Yusty, & Lopez-Hernandez, 2010), which are effective antioxidants and may have particular biological activities. For example, polyphenol-rich extracts and isolated phlorotannin components have been shown to inhibit proliferation of cancer cells (Kwon et al., 2007; Yuan et al., 2005) and to influence anti-inflammatory responses (Kim et al., 2009).

Polyphenols from edible seaweeds have also been suggested to influence responses relevant to diabetes through modulation of glucose-induced oxidative stress (Lee, Han, Heo, Hwang, & Jeon, 2010), as well as through inhibition of starch-digestive enzymes (Lee, Li, Karadeniz, Kim, & Kim, 2008). Indeed, polyphenol-rich extracts from *Ascophyllum* inhibited glucosidase and showed promising anti-diabetic effects in mouse models (Zhang et al., 2007) and

Abbreviations: GAE, gallic acid equivalents; SPE, solid phase extraction; TEAC, Trolox equivalent antioxidant capacity.

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polyphenols from *Ecklonia* have shown positive effects on genetically diabetic mice (Iwai, 2008).

In this study, the polyphenol content of four edible seaweeds was measured and their potential anti-proliferative activity and ability to inhibit starch digestive enzymes (McDougall & Stewart, 2007) was assessed.

2. Materials and methods

2.1. Material and extraction of polyphenols

Dry *Ascophyllum nodosum* was obtained from the Hebridean Seaweed Company (Plot 1.3, Arnish Point Industrial Estate, Isle of Lewis, Scotland) over three years (2007, 2008 and 2010). Dry *Ulva lactuca* (sea lettuce), *Palmaria palmata* (dulse) and *Alaria esculenta* (often referred to as wakame) were obtained from CyberColloids (Strand Farm, Cork, Ireland) in autumn 2007. All samples were supplied air-dried, but were frozen on arrival and freeze-dried at the Scottish Crop Research Institute (SCRI) to <2% residual moisture, as assessed by drying samples to a constant weight at 105 °C. These were ground to powder using a Cyclone Sample mill with 0.1 mm^2 sieves (UDY Corporation, Fort Collins, Colorado). The first extraction was as previously recorded by Yuan et al. (2005). Seaweed samples (25 g) were extracted overnight in 250 ml methanol (100%) on a rotary shaker (180 rpm; Model R100/TW, Rotatest Shaker, Luckham) covered with aluminium foil to protect against light. The extract was subsequently extracted with 200 ml of chloroform and ultra pure water (UPW) in a ratio 1:1 using a separating funnel. All extractions were carried out in duplicate.

Prior to further purification on solid phase extraction (SPE) columns into bound and unbound fractions, the water extracts were concentrated on a rotary evaporator (Buchi Rotavapor, Switzerland) at 45 °C, then acidified with acetic acid prior to SPE. Centrifugation was carried out if necessary to remove insoluble material before evaporation.

The second extraction was performed using 50% acetonitrile/50% UPW containing 0.2% formic acid and used the same proportions of powder and extractant. This simple method has proved effective for extraction of polyphenols from berry samples (McDougall, Kulkarni, & Stewart, 2009). Finally, the third extraction procedure was essentially similar to extraction 2 and only differed in the solid phase extraction procedure.

SPE columns (Giga C18E tubes, Phenomenex Ltd., Macclesfield, UK) were mounted on a suction flask attached to a pump and primed using acetonitrile, washed with UPW then equilibrated with 0.2% (v/v) formic acid. The different seaweed samples were added to separate columns and the run-through collected as the unbound fractions. The column was washed with 0.2% (v/v) formic acid (extraction 2) or UPW (extraction 3) before elution of the bound material with acetonitrile. In some cases, the column was re-equilibrated and the SPE procedure repeated on the unbound material to ensure that all phenolic material was collected. All data provided are based on assays utilising extracts obtained by the third extraction procedure.

2.2. Total phenol content

Samples were assayed for phenol content using a modified Folin–Ciolcateu method (Singleton & Rossi, 1965; Deighton, Brennan, Finn, & Davies, 2000) and the phenol content calculated using a standard curve of gallic acid. Aliquots of each seaweed samples at fixed phenol contents of gallic acid equivalents (GAE) were dried in a Speed Vac (Thermo Fisher, Basingstoke, UK), then stored frozen.

2.3. TEAC assay (Trolox equivalent antioxidant capacity)

This assay was adapted from Deighton et al. (2000). The working ABTS reagent [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid, diammonium salt] at 7 mM was prepared in a ratio of 1:1 with potassium persulphate (33.1 mg/100 ml distilled water). The working reagent was maintained at 30 °C and diluted with ethanol to give an absorbance of ~0.75 at A734. Trolox standard (1 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was prepared in methanol at 25 mg/100 ml. Controls containing ABTS (1 ml) and Trolox (10 μl and 1 ml ABTS) were run at the start of run and at the end of run. Samples (10 μl containing 1 μg GAE) were mixed by the addition of 1 ml ABTS (working solution) at 30 s intervals. Then absorbance was read at A734 exactly after 6 min with water as blank or reference on the CECIL CE 7200 spectrophotometer. The TEAC values were calculated as before (Deighton et al., 2000). Significant difference was assessed using Student's paired *T*-test in the Excel programme.

2.4. Liquid chromatography–mass spectrometry (LC–MS)

Samples (containing 20 μg GAE by Folin assay) were analysed on a LCQ-DECA system, comprising a Surveyor autosampler, pump and photo diode array detector (PDAD) and a ThermoFinnigan mass spectrometer iontrap. The PDAD scanned three discrete channels at 280, 365 and 520 nm. Samples were eluted on a gradient of 5–100% acetonitrile over 30 min on a C18 column (Synergi Hydro C18 with polar end capping, 2 mm × 150 mm, Phenomenex Ltd.) at 200 μl/min. The LCQ-DECA LC–MS was fitted with an electrospray ionisation interface and the samples were analysed in positive and negative mode. There were 2 scan events; full scan analysis, followed by data dependent MS/MS of the most intense ions. The data dependent MS/MS used collision energies (source voltage) of 45% in wideband activation mode. The MS detector was tuned against cyanidin-3-*O*-glucoside (positive mode) and against ellagic acid (negative mode).

2.5. Fractionation on Sephadex LH-20

The procedure was adapted from the Tannins Handbook (see www.users.muohio.edu/hagermae/tannin.pdf) and involves sorption to Sephadex LH-20 in ethanol and selective debinding with aqueous acetone. This is an established method for the separation of tannins from non-tannin phenolics. Firstly, *A. nodosum* powder was extracted as above, but with 50% ethanol rather than acetonitrile, and filtered through a glass microfibre filter (Whatman GF/A). Sephadex LH-20 (GE Healthcare Ltd., London) was swollen in 50% aqueous acetone then poured into a column and equilibrated with three volumes of 50% ethanol. The extract (50 ml) was applied to column and run through with 50% ethanol (and increased to 80% after a column volume) and the combined eluate collected as the unbound LH-20 fraction. The column was then washed with two column volumes of 80% ethanol. Elution with 50% acetone then 80% acetone recovered the bound fraction. The total phenol content of fractions was assayed and evaporated to dryness in suitable aliquots.

2.6. Cell culture and measurements of cell viability

Human colon cancer (Caco-2) cells were grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM), containing D-glucose and L-glutamine (BioWhittaker product No. BE12-604F) and supplemented with 10% fetal calf serum (Gibco Product No. 10500-056), 1% penicillin/streptomycin (Sigma Product No. P-0781), 1% non-essential amino acids (ICN Product No. 1681049) and 25 μg/ml gentamycin (Gibco product No. 15750)