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A comparison of the effects of an *Ascophyllum nodosum* ethanol extract and its molecular weight fractions on the inflammatory immune gene expression *in-vitro* and *ex-vivo*

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ABSTRACT

The anti-inflammatory bioactivities of three crude extracts of *Ascophyllum nodosum* (cold water, hot water and 80% ethanol extract (EE)) were initially evaluated. Following from this, the immune modulatory efficacy of EE and its MW fractions (<3.5, 3.5–100, >100 kDa) were compared. In TNF-α challenged Caco-2 cells, the crude extracts reduced IL-8 production ($P < 0.001$), while the EE caused a >2 fold down-regulation of; cytokines (*IL8*, *TNFA*, *IL1B*, *IL18* and *CSF1*) chemokines (*CXCL10* and *CCL5*), components of NF-κB pathway (*NFKB2* and *IKBKB*), and inflammatory mediators (*PTGS2* and *MIF*) genes. In the porcine colonic tissue *ex-vivo* challenged with lipopolysaccharide, EE and its MW fractions down-regulated the immune related targets *LYZ*, *IL8*, *PTGS2*, *TLR6*, *CXCL10*, *IL6*, *CXCL11*, *ICAM*, *NFKB1* and *CXCL2*. It was concluded that while the MW fractionation potentially generated compounds displaying distinct immune modulatory functions, the crude EE exhibited the most potent immunomodulatory bioactivity.

Industrial relevance: The result demonstrated that the fractionation of crude 80% ethanol extract of *A. nodosum* into MW fractions potentially separate bioactive compounds with distinct immune modulatory function. The crude extract exhibited the most potent immunomodulatory bioactivity in Caco-2 cells challenged with TNF-α and in the porcine colonic tissue challenged with LPS. Such a broad spectrum anti-inflammatory bioactivity of *A. nodosum* ethanol extract can be further explored for its potential application in inflammatory diseases of the mammalian intestine.

1. Introduction

The brown seaweed species, *Ascophyllum nodosum,* is a rich source of bioactive compounds including polysaccharides, proteins and peptides, lipids, pigments and polyphenols (Audibert, Fauchon, Blanc, Hauchard, & Gall, 2010; Holdt & Kraan, 2011). The total polysaccharide content of *A. nodosum* can be as high as 70% on a dry matter basis (Holdt & Kraan, 2011) which predominantly includes cell wall polysaccharides: cellulose and hemicellulose and the storage polysaccharides: alginic acid, fucoidan, mannitol and laminarin (Holdt & Kraan, 2011). The majority of the algal polysaccharides are indigestible within the human gut, produce little or no calories and act as prebiotics that support healthy intestinal gut micro-flora (Holdt & Kraan, 2011; Rioux, Turgeon, & Beaulieu, 2007). *A. nodosum* also contains about 10% protein (on a dry matter basis) and 4.5% lipids (Holdt & Kraan, 2011). While the proteins are rich in essential amino acids, the lipids contain neutral lipids, glycolipids and phospholipids (38.7%, 32.6% and 4.7% of the total lipids, respectively).

A number of health promoting bioactivities of the whole seaweed extracts of *A. nodosum* have been reported; these include anti-oxidant (Abu et al., 2013), anti-inflammatory (Cumashi et al., 2007; Zhang et al., 2014), anti-obesity, probiotic (Dierick, Ovyn, & De Smet, 2009; Gardiner et al., 2008) and immune modulatory properties (Bahar, O'Doherty, Hayes, & Sweeney, 2012) in both *in-vitro* and *in-vivo* models. The whole seaweed extract contains a large number of bioactive chemical compounds that can potentially alter biochemical and physiological processes. However, as a health promoting bioactive, the crude extract has a low functional efficacy due to the presence of relatively low concentrations of the bioactive compounds, influence of the seasonality and growth stage on the concentration of the bioactive compounds

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and presence of chemical contaminants (Colegate & Molyneux, 2008; Holdt & Kraan, 2011). Therefore, crude extracts were fractionated based on polarity and molecular weight in an attempt to isolate distinct groups of bioactives from the whole seaweed extract.

Human colonic adenocarcinoma (Caco-2) cells have been extensively used as a model to study gastrointestinal inflammation (Leonard, Collnot, & Lehr, 2010; Tanoue, Nishitani, Kanazawa, Hashimoto, & Mizuno, 2008). These cells can be induced to differentiate so that they can functionally resemble differentiated human enterocytes (Artursson, 1990). Following challenge with tumor necrosis factor (TNF)-alpha, Caco-2 cells secrete a range of pro-inflammatory cytokines. This principle has been applied previously to identify the immuno-modulatory actions of functional food ingredients, including milk protein hydrolysates (Mukhopadhya et al., 2014) and seaweed extracts (Allsopp et al., 2015). However, the differentiated Caco-2 monolayer lacks the cellular heterogeneity present in the mammalian colonic tissue *in-vivo*. Therefore, the treatment of mammalian intestinal tissue *ex-vivo* is another approach which can be used to investigate the immunomodulatory potential of bioactive food supplements without compromising the complexity of the tissue (Bahar, O'Doherty, Hayes, & Sweeney, 2012; Leonard, Sweeney, Bahar, & O'Doherty, 2012; Smith et al., 2011). This technique involves the dissection of intestinal tissue postmortem, followed by its co-incubation with bacterial lipopolysaccharides and bioactives. Following incubation, the tissue sample is processed for gene expression studies focused on the immune pathways.

The bioactive compounds which are present naturally in whole seaweed extracts are structurally and chemically highly heterogeneous (Cumashi et al., 2007), hence their large-scale isolation and purification for biological testing is challenging. Therefore, relatively crude extracts are often used for biological testing (Colegate & Molyneux, 2008). Presently, there is a lack of information as to whether purified fractions of *A. nodosum* genuinely have advantages over crude fractions in terms of immunomodulatory bioactivity. In light of these challenges, the objectives of this experiment were: 1) to evaluate the anti-inflammatory bioactivity of three crude aqueous extracts of *A. nodosum in-vitro*, 2) to compare the anti-inflammatory mode of action of the most potent crude extract *in-vitro* and *ex-vivo* and 3) to compare the efficacy of the most potent crude extract with that of its three molecular weight fractions (<3.5, 3.5–100, >100 kDa) on the inflammatory immune gene expression in LPS treated porcine colonic tissue *ex-vivo*.

2. Material and methods

2.1. Collection and processing of seaweed

A. nodosum seaweed was harvested off the west coast of Ireland during the winter of 2010. A random selection of fresh samples were harvested from the shore, packed in cool boxes and transported immediately to the laboratory. Samples were washed with tap water to remove sand and epiphytes and then stored at −18 °C. Samples were then freeze-dried and a reference sample was stored in National University of Ireland, Galway, as part of the Marine Functional Food Research Initiative (NutraMara) collection. Samples were subsequently ground into powder using a Waring blender and stored in vacuum-packed bags at −80 °C prior to extraction.

2.2. Preparation of crude extracts and MW fractions

Solubility (crude) extraction of *A. nodosum* ground samples were performed with either: cold water (CWE), hot water (HWE) or ethanol (80%): water (20%) (EE) as solvent following the solid-liquid extractions procedure described earlier (Tierney et al., 2013). Briefly, 200–250 g of freeze-dried powdered samples were mixed with extraction solvent at a ratio of 1:20 *w/v* for cold water and hot water extractions, and at a ratio of 1:10 w/v for ethanol-water extractions. The cold water and ethanol-water extractions were carried out at room temperature in a MaxQ 6000 Shaker (Thermo Fisher Scientific, MA, USA) at 170 rpm. The water extraction carried out at room temperature was termed as cold water extract (CWE) to distinguish from the hot water extraction that was carried out at 60 °C. Cold-water extractions were filtered twice over a 24 h period; ethanol-water extractions were filtered three times through cotton wool and glass wool. The hot water extraction was a sequential extraction carried out on the residue from the cold water extraction at 60 °C. The hot water extract was filtered twice over a 24 h period and the solvent refreshed each time; ethanol was removed from the ethanol-water extract using a rotary evaporator (Heidolph Rotary Evaporator with WB eco bath, Germany) with the water-bath set at 40 °C. All extracts were freeze-dried and the dried extracts were stored at −80 °C until further analysis.

2.3. Preparation of MW fractions

Three MW fractions (<3.5 kDa, 3.5–100 kDa and >100 kDa) of EE were prepared (Tierney et al., 2013) and evaluated for bioactivity in this experiment. The 3.5 kDa cut off was used to predominantly include the low molecular weight tannins that generally have a MW between 0.343 and 3 kDa (Tierney et al., 2013). The next MW cut off range 3.5–100 kDa was expected to include high molecular phlorotannins and/or polysaccharides that have some phlorotannins bound to them, beta-glucans and fucoidan. The high MW (>100 kDa) cut off should include different polysaccharides including the structural polysaccharides. Briefly, the EE was partitioned into hydrophilic and hydrophobic fractions with repeated washing with HPLC-grade water. The washing process was repeated until no further material was removed, leaving behind an oily, highly-pigmented residue, which are presumably mainly carotenoids. The water washes were pooled and the freeze-dried (hydrophilic fraction) which was subjected to the molecular weight cut-off dialysis through 3.5 kDa dialysis tubing. Both, a high molecular weight retentate (>3.5 kDa) and a low molecular weight dialysate (<3.5 kDa) fractions were freeze-dried. The retentate from the 3.5 kDa dialysis was then dissolved in water, added to 100 kDa tubing and dialysis carried out as described previously (Tierney et al., 2013). Both the retentate (>100 kDa) and dialysate (3.5–100 kDa) fractions were freeze-dried.

2.4. In vitro anti-inflammatory assay

Caco-2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corp., San Diego, *CA*, USA) supplemented with 10% (*v*/v) fetal bovine serum (Invitrogen Corp.), 1% (v/v) non-essential amino acids, 1% sodium pyruvate and penicillin (100 U)/streptomycin (100 μg/ml) (All sourced from Sigma–Aldrich Corp., St. Louis, MO, USA). Cells were maintained in vented 75 cm^2 flasks in a humidified cell culture incubator with 5% CO_2 at 37 °C. For the anti-inflammation assay, 10^5 cells/ml were seeded in a 24-well cell culture plate and maintained for 8–10 days. For the treatment, the growth media was removed and the cells were washed with sterile phosphate buffer saline (PBS) and incubated for 3 h in serum and antibiotic free media. To induce a pro-inflammatory response, cells were treated with 10 ng/ml TNF-α. The anti-inflammatory bioactivity of *A. nodosum* preparations were tested through co-treatment of cells with *A. nodosum* solubility extracts at a final concentration of 100, 500 or 1000 μg/ml in the presence of TNF-α. Following 24 h incubation, the media was harvested and the concentration of IL-8 in the supernatants was measured using a human IL-8 sandwich ELISA according to the manufacturer's instructions (R&D Systems Europe, Ltd., Abingdon, UK). Each measurement was performed in triplicate on three independent occasions.