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A cold water extract of *Fucus vesiculosus* inhibits lipopolysaccharide (LPS) induced pro-inflammatory responses in the porcine colon *ex-vivo* model

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ABSTRACT

The objectives of this experiment were to compare the *in-vitro* anti-inflammatory activity of crude extracts (cold water (CWE), hot water and 80% ethanol extract) of *F. vesiculosus* and to predict the key molecular targets of the extract with most anti-inflammatory activity *in-vitro*. In TNF-α challenged Caco-2 cells, of the three crude extracts, the CWE exhibited maximum inhibition of IL-8 production. In the *ex-vivo* challenged porcine colonic tissue, CWE inhibited the expression (>2 fold) of inflammatory mediators (*PTGS2*, *C5*, *LYZ*), cytokines (*IL17A*, *IL8*), chemokines (*CCL2*, *CXCL2*, *CXCL10*, *CXCL11*), cell adhesion molecules (*ICAM1*, *VCAM1*), toll like receptors (*TLR4*, *TLR7*) and components of NF-κB (*NFKB1*, *RELB*), MAPK (*MAP3K8*) and AP-1 (*CJUN*) pathways. The gene expression analysis suggest that the CWE does contain immunomodulatory bioactive compound/s that mediated through the interferon regulatory system 2, TNF-α inducing protein 3 and TNF-α receptor 2 and thus has potential application for human and animal health.

1. Introduction

Fucus vesiculosus, also known as bladder wrack or kelp, is a brown seaweed species widely distributed in the temperate north Atlantic coasts (Jueterbock et al., 2013). Traditionally *F. vesiculosus* is used as a nutritional supplement and believed to have therapeutic potential. *F. vesiculosus* contains high levels of iodine (130 to 160 ppm), hence, an oral intake has been recommended to counteract iodine deficiently (Truus, Vaher, & Taure, 2001). Consumption of the whole *F. vesiculosus* seaweed has reputed health benefits with respect to both cancer and metabolic disease (Gardiner et al., 2008; Gupta & Abu-Ghannam, 2011; Murphy, Hotchkiss, Worthington, & McKeown, 2014). Recently, the potential of *F. vesiculosus* in animal nutrition, with particular focus on gut immune health has been reported (Bahar, O'Doherty, Hayes, & Sweeney, 2012). Despite numerous potential health benefits of *F. vesiculosus*, the molecular mechanisms underlying the bioactivity in mammalian cells/tissues are yet to be elucidated.

F. vesiculosus is a rich source of a range of bioactive compounds including fucoidan, β-glucan, fucoxanthin and phlorotannins (Mohamed, Hashim, & Rahman, 2012; Queiroz et al., 2008; Truus, Vaher, Koel, Mähar, & Taure, 2004). Fucoidan is a polysaccharide composed predominantly of sulfated fucose unit that has reported anti-inflammatory, anti-oxidant and anti-cancer properties (Ale, Maruyama, Tamauchi, Mikkelsen, & Meyer, 2011; Jiao, Yu, Zhang, & Ewart, 2011). Fucoidan inhibited the LPS induced pro-inflammatory response in RAW 264.7 macrophage cells (Kang et al., 2011). It has been reported that the anti-inflammatory activity of fucoidan is mediated through the inhibition of nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), two enzymes important for initiating and sustaining pro-inflammatory cellular responses (Kang et al., 2011). Fucoidan also inhibited the activation of nuclear factor kappa beta (NF-κB) that subsequently reduced systemic levels of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 (Li et al., 2011). Fucoidan also exhibited anti-inflammatory bioactivity through the inhibition of interferon gamma (IFNγ) induced nitrous oxide (NO) production (Do, Kang, Pyo, Billiar, & Sohn, 2010). The bioactive carotenoid present in *F. vesiculosus*, fucoxanthin has been reported to inhibit the LPS induced activation of NF-kB and mitogen activated protein kinases (MAPK) leading to an anti-inflammatory response (Kim et al., 2010). *F. vesiculosus* is also a rich source of polyphenols that exhibit strong anti-oxidant properties. For example, phlorotannin are a complex group of polyphenols found in the brown seaweeds composed of units of the monomer phloroglucinol linked by a range of different chemical bonds. *F. vesiculosus* has recently been reported to contain a vast number of these low molecular phlorotannins (Heffernan, Brunton, FitzGerald, & Smyth, 2015) and exhibit excellent free radical scavenging ability (Mohamed et al., 2012).

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The bioactive compounds extracted from plant materials are generally structurally and chemically highly heterogeneous. Ideally however, structure determination and biological testing of any bioactive compound are best performed on a pure compound (Colegate & Molyneux, 2008). The isolation, purification and subsequent biological testing of single bioactive compounds is often impractical. Therefore, crude extracts are generally applied for biological testing as they are easier and more cost effective to produce. However, these extracts often have a low functional efficacy due to the relatively low concentrations of the bioactive compounds in addition to compositional inconsistency as the bioactive components are influenced by other variables such as seasonal and growth stage (Colegate & Molyneux, 2008). These challenges also apply to the large-scale isolation and purification of *F. vesiculosus* which is technically challenging (Tierney et al., 2014), costly and labor intensive. The initial biological testing of crude extracts, followed by bioactivity guided separation and purification of bioactive compounds remains the main approach for identifying novel bioactive compounds from within natural compounds such as seaweed (Colegate & Molyneux, 2008).

The *ex-vivo* evaluation of bioactive compounds using viable porcine intestinal tissue is a practical and informative technique (Mukhopadhya et al., 2014; Sweeney et al., 2012). A distinct advantage of this technique is that the tissue samples can be challenged directly with inflammatory agents, such as LPS, while still retaining the cellular heterogeneity inherent in the gut, a feature which is lacking in the single cell culture. It has been demonstrated that pig intestinal tissue explants derived from the large intestine can be utilized for the investigation of the immunomodulatory effects of bioactive compounds on the gut epithelium (Bahar, O'Doherty, Vigors, & Sweeney, submitted for publication). This *ex-vivo* technique is a practical solution for screening large numbers of test compounds, an approach which is impractical in live animals. Oral supplementation in live hosts requires the meeting of stringent health and safety as well as ethical guidelines and is limiting in terms of the numbers of samples that can be tested.

It has previously been demonstrated that whole extracts of *F. vesiculosus* inhibited the expression of inflammatory cytokines gene *IL8*, *IL6* and *TNFA* in LPS induced porcine colonic tissue explants (Bahar et al., 2012) suggesting that this seaweed species contains immunomodulatory compounds with potential to treat chronic inflammatory conditions of the gastrointestinal tract of humans and animals. However, a more comprehensive understanding of the cellular and molecular mode of action of the bioactives present in the *F. vesiculosus* extract would aid in its application as a therapeutic agent. Hence, the objectives of this experiment were; a) to compare the *in-vitro* anti-inflammatory activity of different extracts (cold water, hot water and 80% ethanol extracts) of *F. vesiculosus*; b) following identification of the extract with most anti-inflammatory activity *in-vitro*, further analyze the expression of a panel of immune genes in an *ex-vivo* porcine colonic tissue explant culture; and c) predict the key molecular targets of the bioactive components thus elucidating its cellular and biochemical mode of action.

2. Materials and methods

2.1. Collection and processing of seaweed

F. vesiculosus samples were harvested off the west coast of Ireland during the winter of 2010. A random selection of fresh samples were collected, 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 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 ground into powder using a Waring blender and stored in vacuum-packed bags at −80 °C prior to extraction.

2.2. Preparation of solubility extracts

Crude extraction of *F. vesiculosus* ground samples were performed with either cold water (CWE), hot water (HWE) or ethanol (80%): water (20%) (EE) as solvents systems using solid–liquid extractions procedure described previously (Tierney et al., 2013). Briefly, 200–250 g 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. 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 used for further analysis.

2.3. 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) (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 *F. vesiculosus* preparations were tested through co-treatment of cells with *F. vesiculosus* extracts at a final concentration of 1 mg/ml and 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.

2.4. Ex-vivo challenge of colonic tissues

All experimental procedures involving pigs were conducted under experimental license from the Irish Department of Health in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendments of the Cruelty to Animals Act, 1876) Regulation, 1994.

A section of the pig colon (Large White x Landrace; n = 6 pigs) was dissected along the mesentery immediately post-slaughter. The fecal material was removed and the tissue section was washed with sterile PBS. The overlying smooth muscle layer was removed and a colonic section of approximately 1.25 cm × 1.25 cm were transferred into 1 ml DMEM in a 12-well cell culture plate containing 10 μg/ml bacterial lipopolysaccharide (LPS) (*Escherichia coli* serotype O111:B4, Sigma–Aldrich, St. Louis, MO) in the presence or absence of 1 mg/ml *F vesiculosus* extracts. All tissue explants were incubated for 3 h in a humidified cell culture incubator with 5% CO_2 at 37 °C, an *ex-vivo* environment previously found to keep the tissue explant physiologically viable without any adverse affect on the integrity of the mRNA transcripts (Bahar et al., submitted for publication). Following this