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Edible seaweeds' phlorotannins in allergy: A natural multi-target approach

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ABSTRACT

The anti-allergenicity of phlorotannin-targeted extracts from four edible seaweed species of *Fucus* genus was evaluated herein for the first time. Extracts were able to act upon cellular events triggered by immunological reaction (IgE/antigen), and on cellular events downstream the Ca^{2+} influx caused by a chemical stimulus (calcium ionophore A23187), preventing degranulation of RBL-2H3 cells. Furthermore, a dose-dependent behaviour towards allergy-related enzymatic systems was observed for all the phlorotannin extracts. Linear correlations were found between reduction of the allergic mediators released and the total phlorotannin content, as well as between the enzyme inhibition and the amount of phlorotannins in the extracts. These results point to a multi-target anti-allergic capacity of phlorotannin-targeted extracts, which displayed effects on different critical steps of the allergic response, contributing to the valorisation of *Fucus* spp. both as food and for nutraceutical applications.

1. Introduction

Allergies, specifically type I hypersensitivity disorders, are clinically important and increasingly prevalent worldwide (Pawankar et al., 2014). Functionally, allergy is an abnormal adaptive immune response to generally innocuous substances commonly found in the environment. Allergic responses may involve, or not, immunoglobulin E (IgE); however, IgE-mediated reactions are the most prevalent (Galli, Tsai, & Piliponsky, 2008). The molecular and cellular mechanisms behind allergic responses comprise a plethora of preformed and newly synthesized mediators, cell types and pathways. Generally, allergen cross-linking to high-affinity IgE receptors (FcεRI) on mast cells and basophils triggers the beginning of the degranulation process, characterized by a massive release of mediators contained in cell granules (e.g., histamine and β-hexosaminidase), as well as of a wide variety of cytokines, growth and chemotactic factors that are responsible for the clinical symptoms of allergic diseases (Gangwar, Landolina, Arpinati, & Levi-Schaffer, 2017; He, Zhang, Zeng, Chen, & Yang, 2013). Disease management has traditionally relied on allergen avoidance and symptomatic relief (Larsen, Broge, & Jacobi, 2016). With the rise of allergic threats, the regulation of molecular and cellular events (e.g., cell desensitization and inhibition of degranulation) emerges as key strategies to control allergic disorders (Akdis & Akdis, 2015; Larsen et al., 2016). The search for structurally unique molecules with anti-allergic potential will deepen the knowledge on mechanisms underlying allergic events and constitute a major opportunity for drug discovery.

Phlorotannins are phloroglucinol-based compounds found exclusively in brown seaweeds, widely acknowledged to possess a broad spectrum of bioactivities [recently reviewed in (Sanjeewa, Kim, Son, & Jeon, 2016)]. Within the range of marine chemical diversity, they are thought to be among the most promising candidates as anti-allergic compounds. Some studies have addressed the anti-allergic activity of phlorotannins isolated from *Eisenia* and *Ecklonia* genera, showing that they act *via* inhibition of FcεRI expression, calcium influx, and cell degranulation (Ahn et al., 2015; Le, Li, Qian, Kim, & Kim, 2009; Li, Lee, Le, Kim, & Kim, 2008; Shim, Quang-To, Lee, & Kim, 2009; Sugiura et al., 2007, Sugiura, Matsuda, et al., 2006), as well as by the interaction with allergy-related enzymatic systems (e.g., hyaluronidase) (Shibata, Fujimoto, Nagayama, Yamaguchi, & Nakamura, 2002). Some works have also screened the anti-allergic activity of crude seaweed extracts, which was mainly attributed to the presence/content of polyphenol entities, such as phlorotannins (Haider, Li, Lin, Jamil, & Wang, 2009; Samee, Li, Lin, Khalid, & Guo, 2009; Sugiura et al., 2016, 2012, 2009, Sugiura, Matsuda, Okamoto, Kakinuma, & Amano, 2008, Sugiura, Matsuda, et al., 2006). Nevertheless, anti-allergenicity studies focused on seaweed-derived compounds are still scarce, a wide range of

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species around the globe remaining unexplored.

Fucus is one of the richest genus within Fucaceae and considered evolutionary dynamic, with recent radiations of some varieties to species level (e.g., *Fucus guiryi* G.I. Zardi, K.R. Nicastro, E.S. Serrão & G.A. Pearson has recently been elevated from *Fucus spiralis* var. platycarpus) (Zardi et al., 2011). *Fucus* spp. are widely represented on cold and temperate coastal areas, including the Northern Portuguese coastline. In fact, studies devoted to the chemical characterization and assessment of some prominent biological activities of phlorotannin-targeted extracts from edible *Fucus* species have been one of the main interests of our research group (Barbosa et al., 2017; Ferreres et al., 2012; Lopes et al., 2018, Lopes, Pinto, Andrade, & Valentão, 2013, 2012). However, as far as we know, no studies have addressed the anti-allergic potential of phlorotannin extracts from *Fucus* spp.

In this work, we determined the effects of phlorotannin-targeted extracts, obtained from *F. guiryi*, *Fucus serratus* Linnaeus, *F. spiralis* Linnaeus and *Fucus vesiculosus* Linnaeus, on rat basophilic leukemic (RBL-2H3) cell degranulation, experimentally induced by two different stimuli: the calcium ionophore A23187 and the IgE/antigen complex. The released β-hexosaminidase and histamine, both used as degranulation markers, were quantified. In addition, the capacity of the phlorotannin extracts to inhibit hyaluronidase (HAase) and to directly alter β-hexosaminidase activity in the supernatant of degranulated RBL-2H3 cells was screened.

2. Materials and methods

2.1. Standards and reagents

Phloroglucinol (≥99.0%), quercetin (≥95.0%), disodium cromoglicate (DSCG) (≥95.0%), (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), crystal violet staining (CVS), brilliant blue G, 2,4-dimethoxybenzaldehyde (DMBA), 4-dimethylaminobenzaldehyde (DMAB), *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide, dimethyl sulfoxide (DMSO), trypan blue solution, toluene, acetone, sodium hydroxide (NaOH), potassium chloride (KCl), *o*-phthalaldehyde (OPA), *o*-phosphoric acid, citric acid, sodium formate, albumin from bovine serum (BSA), BSA fraction V (7.5% solution), hyaluronic acid (HA) sodium salt from *Streptococcus equi*, HAase from bovine tests (type IV-S; EC 3.2.1.35), as well as anti-2,4-dinitrophenyl monoclonal antibody (anti-DNP IgE) produced in mouse, 2,4-dinitrophenyl albumin (DNP-BSA), and calcium ionophore A23187 (≥98.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, sodium chloride (NaCl), disodium tetraboratedecahydrate ($Na_2B_4O_7{\cdot}10H_2O$), hydrochloride acid (HCl), disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), microcrystalline cellulose for thin-layer chromatography, and formic acid were acquired from Merck (Darmstadt, Germany). Glacial acetic acid was from Fisher Scientific (Loughborough, UK). Water was deionized in a Milli-Q (Millipore, Bedford, MA, USA) water purification system. Dulbecco's Modified Eagle Medium (DMEM)-GlutaMAX™-I, Earle's Balanced Salt Solution (EBSS), heat inactivated fetal bovine serum (FBS), and Pen Strep solution (Penicillin 5000 units/mL and Streptomycin 5000 µg/mL) were obtained from Gibco®, Life Technologies, Invitrogen™ (Grand Island, NY, USA). The rat basophilic leukaemia cell line RBL-2H3 was purchased from the American Type Culture Collection (ATCC®) (LGC Standards S.L.U., Spain).

2.2. Macroalgae samples

Fucales used in this work were randomly collected during low-tide periods from different rocky shores of Northern Portugal. *Fucus guiryi* G.I. Zardi, K.R. Nicastro, E.S. Serrão & G.A. Pearson, and *Fucus serratus* Linnaeus were harvested from Praia da Amorosa (41°26′N, 8°49′22″W), whereas both *Fucus spiralis* Linnaeus, and *Fucus vesiculosus* Linnaeus were from Praia Norte (41°41′49″N, 8°51′08″W). With the exception of *F. vesiculosus*, collected in 2015, all the other *Fucus* specimens were collected in 2013. After collection, samples were placed on ice and immediately transported to the laboratory in insulated sealed ice-boxes, to protect them from heat, air and light exposure. The fresh biomass was cleaned and thoroughly washed with NaCl aqueous solution (3.5%, *w/w*) to remove epiphytes and encrusting material. All samples were kept at −20 °C, prior to freeze-drying in a Virtis SP Scientific Sentry 2.0 apparatus (Gardiner, NY, USA). Each sample corresponds to a pool of branches from, at least, five adult individuals in the same stage of development. Thereafter, the dried material was pulverized (particle size ≤ 910 µm) and stored in a desiccator, in the dark, until phlorotannin extraction. No alteration (color, smell, humidity) was observed during storage.

2.3. Phlorotannin extraction, purification and quantification

Phlorotannin-targeted extracts were prepared following a well-established protocol (Barbosa et al., 2017; Ferreres et al., 2012; Lopes et al., 2013, 2012). The extraction procedure involved several steps and each of them was carefully performed, protected from light and at room temperature. Briefly, each *Fucus* sample (ca. 1 g of powdered lyophilized material) was subjected to aqueous acetone extraction. The obtained crude extracts were purified with microcrystalline cellulose, which was thoroughly washed with toluene to remove lipophilic components and then rinsed with a mixture of acetone:water (7:3, *v/v*) for phlorotannin release. To prepare stock phlorotannin working solutions, the purified extracts were evaporated until complete dryness, and the resulting dried residue reconstituted in DMSO at a final concentration of 100 mg dry extract/mL. All stock solutions were properly stored at −20 °C until analytical and biological determinations.

The amount of phlorotannins in each purified *Fucus* spp. extract was spectrophotometrically determined at 515 nm (Lopes et al., 2012) through the specific reaction between DMBA and 1,3- and 1,3,5-substituted phenols to form a colored product (Stern, Hagerman, Steinberg, Winter, & Estes, 1996), from a standard calibration curve ($y = 0.0215x + 0.0149$; $r^2 = 0.9997$) of the phlorotannin building block, phloroglucinol. Phlorotannin content was expressed as µg of phloroglucinol equivalents (PGE)/100 mg of dry extract.

2.4. Cell culture

RBL-2H3 cells (passage range 12–22) were cultured in DMEM + GlutaMAX™ – I supplemented with 10% FBS and 1% Pen Strep solution and maintained at 37 °C in a -humidified atmosphere with 5% CO_2. At near confluence (80–90%), cells were seeded at 3×10^5 cells/mL in 24-wells plates for experiments, as outlined below.

2.5. Cell assays

Cell assays were conducted as before (Pinho, Sousa, Valentao, Oliveira, & Andrade, 2014), with some modifications. The calcium ionophore A23187 and an immunologic stimulus referred herein as IgE/antigen were used to induce degranulation. The effect of the extracts in the absence of stimuli was simultaneously determined for each experiment. The maximum non-interfering DMSO concentration was determined, and 0.5% (*v/v*) was never exceeded. Quercetin was used as positive anti-degranulation control.

2.5.1. A23187-mediated cell degranulation

Cells were pre-treated with serial dilutions of each purified phlorotannin extract (in EBSS + 0.1% BSA) 30 min prior to the addition of the ionophore (150 ng/mL), and maintained in culture for another 30 min. Afterwards, supernatants were collected and used for the determination of both β-hexosaminidase and histamine release, whereas MTT viability and CVS proliferation assays were performed in adherent cells.