Algal Research 28 (2017) 1–8

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Phlorotannin extracts from Fucales: Marine polyphenols as bioregulators engaged in inflammation-related mediators and enzymes

Mariana Barbosa[a], Graciliana Lopes[a], Federico Ferreres[b], Paula B. Andrade[a], David M. Pereira[a], Ángel Gil-Izquierdo[b], Patrícia Valentão[a,*]

[a] REQUIMTE/LAQV, Laboratório de Farmacognosia, Departamento de Química, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

[b] Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS (CSIC), P.O. Box 164, 30100 Campus University Espinardo, Murcia, Spain

ARTICLE INFO

Keywords:
Fucales
Phlorotannins
Anti-inflammatory
Lipoxygenase
Nitric oxide
RAW 264.7 cells

ABSTRACT

Phlorotannins are widely recognized as an inexhaustible family of naturally occurring molecules, with great potential in food and pharmaceutical industries. The present work explores the anti-inflammatory activity of phlorotannins from different *Fucus* species native from the Northern Atlantic coast, and from *Fucus vesiculosus* Linnaeus grown in an integrated multi-trophic aquaculture (IMTA) system, all scarcely studied regarding these compounds. Purified phlorotannin extracts were evaluated for their toxicity and anti-inflammatory potential in both cell (RAW 264.7 macrophages) and cell-free systems [lipoxygenase (LOX) inhibition and nitric oxide radical (·NO) scavenging activity]. Phlorotannin content ranged between 110.28 and 288.36 μg of phloroglucinol equivalents (PGE)/100 mg dry purified extract (DE), wild species presenting higher content than those from IMTA. Regarding LOX, a strong correlation ($r = -0.9118$; $p < 0.0001$) between the IC_{50} values and total phlorotannin content was found, *Fucus guiryi* G.I. Zardi, K.R. Nicastro, E.S. Serrão & G.A. Pearson exhibiting the best inhibitory capacity (IC_{50} = 82.10 μg/mL), while *F. vesiculosus* from IMTA was the less effective (IC_{50} > 500 μg/mL). Correlation was also observed for ˙NO scavenging ($r = -0.6363$; $p = 0.0108$). The anti-inflammatory capacity of phlorotannin extracts was further evaluated using RAW 264.7 macrophages stimulated with bacterial lipopolysaccharide, as model of inflammation. The extracts studied were not toxic at the tested concentrations (31.25–500 μg/mL). *F. vesiculosus* from wild origin was the most effective in reducing NO in cell culture medium (IC_{25} = 56.52 μg/mL), closely followed by other seaweed species under study. The IMTA species was the less effective one (IC_{25} = 317.41 μg/mL). According to the results obtained, phlorotannin extracts from Fucales arise as potentially beneficial in inflammation-related conditions, effectively acting upon enzymatic and non-enzymatic inflammatory targets.

1. Introduction

Due to its wealth of biodiversity, marine world has arisen curiosity and interest in several scientific areas, marine organisms-related research experiencing an exponential growth over the last years. Macroalgae, commonly addressed as seaweeds, are recognized as one of the most prolific group of marine organisms in terms of bioactive molecules. They thrive in complex habitats and are often exposed to fluctuating environmental conditions, making these photosynthetic organisms invaluable producers of diverse and structurally complex bioactive compounds [1]. Within seaweed groups, the brown ones (Ochrophyta) stand out, not only for their widespread distribution, but also because of their exclusive metabolic capacity to biosynthesize phlorotannins. Chemically, these compounds result from the polymerization of the aromatic precursor phloroglucinol (1,3,5-trihydroxybenzene), through the polyketide pathway. Six different classes of phlorotannins can be distinguished (phlorethols, fuhalols, fucols, fucophlorethols, eckols and carmalols) according to the nature of the structural linkages between phloroglucinol units, and the number and distribution of hydroxyl groups [2]. These polyphenolic entities have a wide range of molecular sizes (126 Da–650 kDa), occurring mainly in the epidermal cortex of seaweeds (from 0.5 up to 20% of algal dry weight) [3]. Significant fluctuations of total phlorotannin content in the different algae orders are well documented, the variations appearing to be related with both biotic and abiotic factors, such as temperature, light intensity exposure, nutrient availability, time of harvest and the

* Corresponding author.
E-mail address: valentao@ff.up.pt (P. Valentão).

http://dx.doi.org/10.1016/j.algal.2017.09.009
Received 19 April 2017; Received in revised form 5 September 2017; Accepted 8 September 2017

presence of predators in the surrounding waters [4,5]. The Northern Atlantic region, including the Northern Portuguese coast, represents a hotspot of marine diversity. Though considerable efforts have been made on the search of functional compounds from some native macroalgae species harvested and cultivated in the Portugal coast, several others remain unexplored [6]. Few surveys report the biological activities of phlorotannins from native brown macroalgae of this region of the globe, the majority of them being conducted by our research group [7–10].

Within the broad spectrum of biological activities already described for phlorotannins, their anti-inflammatory potential has attracted particular attention [8,11–17]. In inflammatory processes, pro-inflammatory cytokines and other stimuli lead to the expression of the inducible nitric oxide synthase in monocytes/macrophages, neutrophil granulocytes and many other cells [18]. As consequence, large amounts of nitric oxide (NO) are synthesized, greatly exceeding the physiological NO production. Concomitantly, during this process arachidonic acid levels are also considerably increased and, thus, more prone to be enzymatically transformed into potent inflammatory mediators that exert their effects *via* binding to specific membrane and nuclear receptors [19]. Among the most relevant enzymes involved in this process, lipoxygenases (LOX) are highlighted. This family of enzymes is crucial for leukotriene biosynthesis from arachidonic acid and, consequently, one of the main contributors for the progression of inflammation. Hence, interference with these targets constitutes an attractive strategy for the development of anti-inflammatory agents. Although a significant decrease of NO levels has already been addressed for phlorotannin extracts and/or isolated compounds [8,12,13,15,17], evidence of LOX inhibitory potential of phlorotannins is still scarce [20,21].

This work aimed to evaluate the anti-inflammatory potential of phlorotannin purified extracts of four Fucales species, widely represented on the Portuguese coastline and still underexplored in this field, using a multiple-method approach of well-documented *in vitro* cell and cell-free assays.

2. Materials and methods

2.1. Standards and reagents

Phloroglucinol (≥ 99.0%), lipoxidase from *Glycine max* (L.) Merr. (type V-S; EC 1.13.11.12), linoleic acid (≥ 99.0%), dexamethasone (≥ 97.0%), (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue solution, sodium nitroprusside dehydrate (SNP), sulfanilamide, 2,4-dimethoxybenzaldehyde (DMBA), lipopolysaccharide (LPS) from *Escherichia coli*, toluene, dimethyl sulfoxide (DMSO), and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, cellulose microcrystalline for thin-layer chromatography, *N*-(1-napthyl) ethylenediamine, acetic acid (glacial), hydrochloride acid (HCl), and di-sodium hydrogen phosphate dehydrate were acquired from Merck (Darmstadt, Germany). Water was treated in a Milli-Q (Millipore; Bedford, MA, USA) water purification system. Dulbecco's Modified Eagle Medium (DMEM)-GlutaMAX™-I, Hanks' balanced salt solution (HBSS), heat inactivated foetal 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 murine macrophage-like cell line RAW 264.7 was purchased from the American Type Culture Collection (LGC Standards S.L.U., Spain).

2.2. Sampling

Macroalgae used in this work consist of four Ochrophyta species randomly collected during low-tide periods from different places of the Western coast of Portugal (Fig. 1): *Fucus guiryi* G.I. Zardi, K.R. Nicastro, E.S. Serrão & G.A. Pearson (*Fg*), *Fucus serratus* Linnaeus (*Fser*), *Fucus spiralis* Linnaeus (*Fspi*), and *Fucus vesiculosus* Linnaeus (*Fves-w*). Specimens were identified based on overall morphology: *Fg*, *Fspi* and *Fves* were distinguished as described in [22]; *Fser* identification followed the criteria established in [23]. The collection sites are located in Northern Portugal, where sea surface temperature ranges from around 11 °C in the winter to around 22 °C in the summer, and coastal upwelling events are present, with maxima from July to September [24,25]. *F. vesiculosus* was also harvested in an integrated multi-trophic aquaculture (IMTA) system (*Fves-a*) and supplied by ALGAplus, a company devoted to the production of macroalgae and by-products in controlled environment and with biological certification. Macroalgal samples commercialized by ALGAplus are produced at “Ria de Aveiro”, located in the Northwest of Portugal, and connected to the Atlantic Ocean through two major rivers, Antuã and Vouga.

The genus *Fucus* is widely represented on the Portuguese coastline, *F. guiryi* being only recently elevated from variety (*F. spiralis* var. *platycarpus*) to species level [22].

The collected material was 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. Each macroalgae sample corresponds to a mixture of branches from, at least, five adult individuals in the same stage of development. All samples were kept at − 20 °C, prior to freeze-drying in a Virtis SP Scientific Sentry 2.0 apparatus (Gardiner, NY, USA). The dried material was powdered (< 910 μm) and kept in the dark, in a desiccator, until it was subjected to phlorotannin extraction. No sample alteration (colour, smell, humidity) was observed during storage.

2.3. Purified phlorotannin extract preparation

Phlorotannin extracts were obtained as previously reported [8]. Briefly, *ca.* 1 g of powdered lyophilized material was subjected to aqueous acetone extraction. The crude extracts were purified with cellulose, which was then thoroughly washed with toluene. Afterwards, cellulose was rinsed with acetone:water (7:3, *v/v*) to release the phlorotannins, and the filtrate was evaporated until complete dryness. To prepare stock phlorotannin working solutions, the obtained extracts were reconstituted in DMSO at a final concentration of 100 mg of dry purified extract (DE)/mL. The extraction yields ranged between 15.0 and 22.5 mg DE/100 g dry algal powder.

2.4. Phlorotannin quantification

The phlorotannin content of the purified *Fucus* sp. extracts was spectrophotometrically determined by the specific reaction between DMBA and 1,3- and 1,3,5-substituted phenols to form a coloured product, as before [8]. The amount of phlorotannins in each purified extract was determined from a standard calibration curve ($y = 0.0213x + 0.0131$; $r^2 = 0.9997$) with serial dilutions of phloroglucinol (1.2–150 μg/mL). The results are expressed as μg of phloroglucinol equivalents (PGE)/100 mg DE. Phlorotannin content was calculated from five independent determinations and the results are shown as mean ± standard deviation (SD).

2.5. Cell-free assays

2.5.1. Lipoxygenase inhibition

The inhibitory effect on LOX was assessed in 96-well plates, based on a procedure recently employed [26]. The tested extracts were pre-incubated with the enzyme in buffer for 5 min at room temperature, before adding substrate (linoleic acid) to start the reaction. Absorbance was measured continuously at 234 nm, for 3 min, on a Synergy™ HT plate reader (Biotek Instruments; Winooski, USA) operated by Gen5 Software. The maximum non-interfering DMSO concentration was determined, and 0.5% (*v/v*) was not exceeded. LOX inhibition was