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Profiling phlorotannins from *Fucus* spp. of the Northern Portuguese coastline: Chemical approach by HPLC-DAD-ESI/MS^n and UPLC-ESI-QTOF/MS

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

[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

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ABSTRACT

Several attempts have been made in recent years to fully characterize phlorotannin-rich extracts. Nevertheless, this remains a challenging quest, not only because of seaweed complex chemical composition, but also due to the diverse structural assemblage within phlorotannin molecules (i.e., type of linkage, modification site, and number of additional hydroxyl groups). In this work, 22 phlorotannins were tentatively identified and their presence confirmed, in purified extracts obtained from four *Fucus* species, by HPLC-DAD-ESI/MS^n and UPLC-ESI-QTOF/MS analyses. The characterized phlorotannins exhibited molecular weights ranging from 370 to 746 Da and relatively low degree of polymerization (3–6 phloroglucinol units). Isomers of fucophlorethol, dioxinodehydroeckol, difucophlorethol, fucodiphlorethol, bisfucophlorethol, fucofuroeckol, trifucophlorethol, fucotriphlorethol, tetrafucophlorethol, and of fucotetraphlorethol were identified. Among the analysed phlorotannin purified extracts, only the ones wild-sourced and from aquaculture-grown *Fucus vesiculosus* exhibited five and six-ringed phloroglucinol oligomers in their composition. The remaining extracts from *Fucus guiryi*, *Fucus serratus* and *Fucus spiralis* were richer in both trimers and tetramers. The variability observed for the overall phlorotannin composition points to the influence of species-specific and/or external factors. As far as we know, mass spectra confirming the presence and degree of polymerization of phlorotannins in Portuguese-sourced brown macroalgae, specifically *F. guiryi* and *F. serratus*, as well as of aquaculture-grown *F. vesiculosus*, are not found in literature. The information gained in this work ascertains the role of advanced analytical tools in facilitating the use of this valuable natural resource for the development of macroalgal-based products, opening doors for future application of phlorotannin-rich extracts in the commercial areas related to nutraceuticals, pharmaceuticals, and cosmetics.

1. Introduction

An extremely variable and often complex molecular assemblage from the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) gives origin to an almost inexhaustible family of naturally occurring polyphenolic entities: phlorotannins. These compounds exhibit a wide range of molecular weights (126 Da–650 kDa) and are biosynthesized exclusively by brown seaweeds (Ochrophyta), *via* the acetate-malonate pathway [1]. The amount and the degree of polymerization of phlorotannins are highly variable among seaweed species, and deeply affected by surrounding factors (e.g., UV radiation) [2], reinforcing their pivotal role as primary metabolites with transitional secondary biological functions in brown macroalgae [3].

Over the last decade, studies on the biological activities of phlorotannins have increased exponentially, and a growing commercial interest into their potential application in a range of therapeutics has arisen [4,5]. Nevertheless, some obstacles have hampered the development of phlorotannin-based products (e.g., difficulties in separation and purification steps, and subsequent characterization due to their similar polarity and polymeric structure) [6]. Six different classes of phlorotannins (phlorethols, fuhalols, fucols, fucophlorethols, eckols and carmalols) can be established according to the nature of the structural

* Corresponding authors.
E-mail addresses: pandrade@ff.up.pt (P.B. Andrade), federico@cebas.csic.es (F. Ferreres).

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linkages between phloroglucinol units, as well as to the number and distribution of hydroxyl (OH) groups [4]; however, within each class, it is still possible to have both structural and conformational isomers, increasing the complexity and variability of these molecules [7]. In a general way, phlorethols and fuhalols are characterized by the presence of aryl-ether linkages between phloroglucinol units; however, fuhalols exhibit a regular sequence of *para*- and *ortho*- ether bonds, as well as additional OH groups in every third ring, and one or more OH groups in the whole molecule. Fucols, on the other hand, consist of phloroglucinol monomers linked by aryl-aryl bonds, while fucophlorethols present a mixture of ether and phenyl linkages between the basic unit. The generally low molecular weight of eckols and the presence of a phenoxyl substitution at C4 are key-structural motifs that differentiate eckols from carmalols, both characterized by the presence of dibenzodioxin linkages [4,8].

Phlorotannins have been conventionally determined on crude extracts as the total amount of phenolic compounds, using non-specific spectrophotometric-based assays like Folin-Ciocalteu [9]. Another quantitative colorimetric method, employing 2,4-dimethoxybenzaldehyde (DMBA) that reacts specifically with 1,3- and 1,3,5-substituted phenols to form a coloured product, has also been employed with good repeatability and high precision [8,10]. Although these methods are quite simple to use, giving a general estimation of the amount of phlorotannins in the extract, they provide no information on the qualitative phlorotannin profile. Chromatographic techniques arise then as a powerful tool for the analysis of these algal constituents, and a few recent studies employing advanced liquid chromatography-mass spectrometry (LC-MS) methods have achieved, at least in part, the characterization of phlorotannins [6,7,9,11–16]. More than 150 phlorotannins were identified in several brown seaweed species [8], but the noteworthy structural heterogeneity and complexity of these compounds makes their profiling an almost unlimited field of research.

The aim of this work was to establish the phlorotannin composition of four *Fucus* species (*Fucus guiryi* G.I. Zardi, K.R. Nicastro, E.S. Serrão & G.A. Pearson, *Fucus serratus* Linnaeus, *Fucus spiralis* Linnaeus, and *Fucus vesiculosus* Linnaeus) widely represented in the Northern Portuguese coastline. Some studies have previously characterized phlorotannins found in *Fucus* species, such as *F. vesiculosus* [9,12,13,15,17], *F. spiralis* [6,7,9,11] and *F. serratus* [13], from different regions of the globe. To the best of our knowledge, this is the first study confirming the presence and degree of polymerization of phlorotannins in *F. guiryi*. Moreover, as macroalgal cultivation in integrated multi-trophic aquaculture (IMTA) systems has become more widespread, there is a need to expand the knowledge on this material [18,19]. Therefore, *F. vesiculosus* grown in IMTA was also analysed. High-performance liquid chromatography-diode array detection coupled to tandem electrospray ionization mass spectrometry (HPLC-DAD-ESI/MS^n) and ultra-performance liquid chromatography-electrospray ionization coupled to quadrupole time-of-flight high-definition mass spectrometry (UPLC-ESI-QTOF/MS) were employed to provide evidence of the presence of phlorotannins, their varying degree of polymerization and tentative identification in purified extracts of *Fucus* spp.

2. Materials and methods

2.1. Standards and reagents

All solvents were of HPLC-grade. Formic acid, phloroglucinol (≥ 99.0%) and 2,4-dimethoxybenzaldehyde (DMBA) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol, acetonitrile, glacial acetic acid and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Table 1
Sample characterization.

Sample	Species	Origin	Collection date
Fg	*F. guiryi*	Praia da Amorosa (N 41°41′49.75, W 8°51′3.52)	Dec 2013
Fser	*F. serratus*	Praia da Amorosa (N 41°41′49.75, W 8°51′3.52)	Dec 2013
Fspi	*F. spiralis*	Praia Norte (N 41°38′51.44, W 8°49′32.53)	Dec 2013
Fves-w	*F. vesiculosus*	Praia Norte (N 41°38′51.44, W 8°49′32.53)	May 2015
Fves-a	*F. vesiculosus*	IMTA (N 40°36′45.27, W 8°40′26.88)	Jul 2016

2.2. Sampling, phlorotannin extraction and quantification

Brown macroalgae (Ochrophyta) of the order Fucales used in this work were randomly collected during low-tide periods from different rocky shores of Northern Portugal (Table 1): *F. guiryi* (*Fg*), *F. serratus* (*Fser*), *F. spiralis* (*Fspi*), and *F. vesiculosus* (*Fves-w*). Of the selected species, *F. vesiculosus* was also grown in an integrated multi-trophic aquaculture (IMTA) system (*Fves-a*), and supplied by ALGAPlus (Ílhavo, Portugal). The collected samples, consisting of, at least, five individuals in the same stage of development, were washed to remove epiphytes, freeze-dried in a Virtis SP Scientific Sentry 2.0 apparatus (Gardiner, NY, USA) and ground to a fine powder (particle size ≤ 910 μm).

The extracts were prepared with approximately 1 g of powdered lyophilized material, using 10 mL of methanol:water (1:1, v/v), under the following conditions: 1 h of sonication, 24 h maceration at room temperature, followed by 1 h of sonication. Afterwards, the extracts were centrifuged (10,000 rpm, 10 min) and the methanol present in each supernatant was removed in a Savant™ SPD121P SpeedVac™ Concentrator (Thermo Scientific, Alcobendas, Spain). The remaining aqueous mixture was loaded onto a Sep-Pak C18 Plus Short Cartridge (360 mg sorbent per cartridge, 55–105 μm particle size, 50/pk [WAT020515]) (Waters, Milford, MA, USA), which had been pre-conditioned with methanol followed by water, and then washed with water. Phlorotannins were eluted with methanol and the solvent was evaporated under reduced pressure until complete dryness. The resulting phlorotannin-rich fraction was resuspended in a mixture of methanol:water (1:1, v/v) and filtered through a 0.45 μm pore size membrane (Millipore) before analysis.

The phlorotannin content of the *Fucus* spp. 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 [11]. The amount of phlorotannins in each extract was determined from a standard calibration curve ($y = 0.0233x + 0.0125$; $r^2 = 0.9995$) with serial dilutions of phloroglucinol (2.3–75 μg/mL) and expressed as mean ± SD (mg phloroglucinol equivalents (PGE)/kg dry algae) of 3 independent experiments performed in duplicate.

2.3. HPLC-DAD-ESI/MS^n qualitative analyses

Chromatographic analyses were performed in an Agilent HPLC 1200 series equipped with a diode array and mass detectors in series (Agilent Technologies, Waldbronn, Germany), as previously described [20]. The HPLC consisted of a binary pump (model G1376A), an autosampler (model G1377A) refrigerated at 4 °C (G1330B), a degasser (model G1379B), and a diode array detector (model G1315D). The HPLC system was controlled by ChemStation software (Agilent, v. B.01.03-SR2). The mass detector was a Bruker ion trap spectrometer (model HCT Ultra) equipped with an electrospray ionization interface and was controlled by LCMSD software (Agilent, v. 6.1). Briefly, extract (20 μL) elution was carried out at a flow rate of 0.8 mL/min, on a Kinetex column (5 μm, C18, 100 Å, 150 × 4.6 mm; Phenomenex, Macclesfield,