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Anti-proliferative activity and chemical characterization by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry of phlorotannins from the brown macroalga *Sargassum muticum* collected on North-Atlantic coasts

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ABSTRACT

In the present work, the phlorotannin composition of different *Sargassum muticum* samples collected at different locations along the North Atlantic coasts as well as the bioactivities related to these components were investigated. After pressurized liquid extraction, the samples collected at the extreme locations of a latitudinal gradient from Portugal and Norway, were found to be the richest on total phenols and, particularly, on phlorotannins, containing up to 148.97 and 5.12 mg phloroglucinol equivalents g^{-1}, respectively. The extracts obtained from these locations were further purified and chemically characterized using a modified HILIC × RP-DAD-MS/MS method. The application of this methodology allowed the tentative identification of a great variability of phlorotannins with different degrees of polymerization (from 3 to 11) and structures, determined for the first time in *S. muticum*. The most-abundant phlorotannins on these samples were fuhalols, hydroxyfuhalols and phlorethols, showing also particularities and important differences depending on the geographical location. Afterwards, the antiproliferative activity of these extracts against HT-29 adenocarcinoma colon cancer cells was studied. Results revealed that the richest *S. muticum* samples in terms of total phlorotannins, i.e., those from Norway, presented the highest activity, showing a good cytotoxic potential at concentrations in the medium micromolar range.

1. Introduction

Phlorotannins are polyphenolic compounds widely recognized to be exclusive from brown seaweeds (Phaeophyceae) [1]. This particular type of polyphenols comprises a very heterogeneous group of polymeric compounds with a great chemical variability [2]. The interest of these compounds is related to their associated bioactivities, such as antioxidant [3–5], anti-inflammatory [6], anti-bacterial [7,8], antidiabetic [9] or anti-adipogenic [10], among others. Moreover, their potential anti-proliferative activity has been pointed out by several researches [11–13]. Phlorotannin content in brown algae can reach up to 15% of dry weight, depending on species, and they may be found in free form or forming complexes with different components of the cell walls, such as alginic acid [14]. From a purely chemical point of view, phlorotannins are made up of phloroglucinol (1,3,5-trihydroxybenzene) units with varying degrees of polymerization that may be linked through different bonds forming several structures and types, namely: fuhalols and phlorethols, which contain ether linkages; fucols, with phenyl linkages; fucophlorethols in which both ether and phenyl linkages are present; and eckols, that possess a benzodioxin linkage. Although their presence in brown algae is widely accepted, it is rather difficult to find studies in which the complete characterization of such complex polymeric structures is carried out. In fact, several

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approaches have been attempted for the structural elucidation of phlorotannins in their native form; for instance, Stiger-Pouvreau et al. [15] employed one- and two-dimensional nuclear magnetic resonance (NMR) (1H, heteronuclear multiple bond correlation) together with *in vivo* NMR (high-resolution magic-angle spinning, HR-MAS NMR) analyses, to structurally elucidate and fingerprint phlorotannin signals in different Sargassaceae species. Results revealed that these techniques were useful for discriminating among species, giving a differentiated profile but only determining the class of phlorotannins in the sample, without elucidating the entire structure of any compound. In a recent work carried out in our laboratory [16], a new comprehensive two-dimensional liquid chromatography coupled to DAD and tandem mass spectrometry (LC × LC-DAD-MS/MS) methodology was developed based on the coupling of a hydrophilic-interaction chromatography (HILIC)-based separation in the first dimension and an RP-based separation in the second dimension that allowed the separation and identification of more than 50 compounds in a *Cystoseira abies-marina* brown algal extract. By using this approach, phlorotannins containing from 5 to 17 phloroglucinol units were identified in this sample [16]. The application of this methodology to *S. muticum* could therefore imply a definitive step forward for the characterization of its phlorotannin composition.

S. muticum is an invasive brown macroalga widely spread along the European Atlantic coasts [17] Although native from Japan, this macroalga grows well in a variety of different environments, being in fact, one of the most readily available Sargassaceae species in Europe. Considering its availability and the fact that the presence of phlorotannins in *S. muticum* composition has been already confirmed [5,8], this seaweed has been pointed out as a potential sustainable source of bioactive compounds.

Different methods have been tested to extract phlorotannins from brown algae; the classical procedure [1] involves a solid–liquid extraction with large volumes of aqueous mixtures of ethanol or methanol for a long time. New green processes have been previously shown to be suitable for the extraction of bioactive compounds from a variety of different natural sources [18]; among them, centrifugal partition extraction (CPE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) have been employed, and compared to classical solid-liquid extraction, to obtain bioactive phenolic compounds from *S. muticum* [5]. Results demonstrated that PLE can be employed with advantages for obtaining extracts rich in phenolic compounds from brown algae, with high efficiency and complying with the rules of green chemistry. On the other hand, in a recent work carried out in our laboratory, enzyme-assisted extraction (EAE) was studied and compared to an optimized PLE process to try to increase the recovery of phenolic compounds from *S. muticum* [19]. This study showed that EAE did not significantly improve the results directly attainable through the use of PLE.

Thus, in the present work, the previously optimized PLE process [19] was applied to the extraction of phlorotannins from *S. muticum* samples collected at 13 different locations along the North-Atlantic coasts (Portugal, Spain, France, Ireland and Norway) with the aim to study the influence of the growing conditions on the chemical composition of the extracts. The extracts were characterized in terms of total phenol content, total phlorotannin content and antioxidant activity. Besides, a comprehensive two-dimensional liquid chromatography (LC × LC) method was optimized and applied to the richest samples to chemically characterize for the first time the native complex phlorotannin composition of *S. muticum*. Moreover, these extracts were also assayed to test their potential anti-proliferative activity against human colon cancer cells.

2. Materials and methods

2.1. Samples and chemicals

Samples of the brown alga *S. muticum* were collected from April to May 2011 in 13 different sites of five European Atlantic coast countries (Portugal, Spain, France, Ireland and Norway) as already described in Tanniou et al. [8]. The algae were rinsed firstly with filtered seawater and then with distilled water to remove the residual sediments and salts. After that, the samples were dried with absorbent paper and cut into fragments before their freeze-drying. Finally the dry material was powdered and sieved at 250 μm.

The solvents employed were HPLC-grade. Acetonitrile, ethanol, methanol and acetone were acquired from VWR Chemicals (Barcelona, Spain), whereas dichloromethane was acquired from Fluka AG (Buchs, Switzerland) and ethyl acetate from Scharlau (Barcelona, Spain). Ultrapure water was obtained from a Millipore system (Billerica, MA, USA).

Gallic acid, phloroglucinol, acetic acid, formic acid, 2,4-dimethoxybenzaldehyde (DMBA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Sigma–Aldrich (Madrid, Spain). The Folin–Ciocalteu phenol reagent was provided by Merck (Darmstadt, Germany). Hydrochloric acid was obtained from Probus (Barcelona, Spain). For inhibition of cell proliferation assays, dry purified extracts were dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich) at the appropriate concentrations and stored as aliquots at −80 °C until use.

2.2. Pressurized liquid extraction (PLE)

Firstly, extractions of freeze-dried and ground *S. muticum* samples from 13 different localizations along the North-Atlantic coasts were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. For each extraction, an 11 mL stainless steel extraction cell was employed to load the sample. The extraction cell bottom was loaded with 1 g of sea sand, followed by 1 g of dried brown alga being mixed with the same quantity of sea sand. Subsequently, 1 g of sea sand was added on top as dispersive agent. Before the static extraction period, an instrumentally preset warming-up time of 6 min was used. The extraction conditions applied were based on a previous optimization [19], including the use of ethanol:water (95:5) as extraction solvent at 160 °C and 10.3 MPa for 20 min. Each extraction was carried out by duplicate. After the extraction process, the ethanol was removed by evaporation (Rotavapor R-210, Buchi Labortechnik AG, Flawil, Switzerland) and finally, the extracts were freeze-dried (Labconco Corporation, MO) and kept in the darkness at −20 °C until analysis.

2.3. Phlorotannins purification procedure

In order to obtain concentrated phlorotannin extracts, a purification protocol previously reported by Stiger-Pouvreau et al. [15] was applied to the *S. muticum* samples from Norway and Portugal. The dried extracts were re-dissolved in water and submitted to a liquid-liquid extraction with dichloromethane (1:1, *v/v*) in order to eliminate the lipidic compounds and chlorophylls present in the extract, repeating this step several times until a colorless non-polar fraction was obtained. After that, successive precipitations of proteins and carbohydrates were carried out with acetone and ethanol, respectively, ending with the elimination of the organic solvent using a gentle stream of nitrogen. Finally, phlorotannins were extracted from the water fraction with three rinses with equivalent volumes of ethyl acetate. The ethyl acetate fractions