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Angiotensin I-converting enzyme (ACE) inhibitory activity of *Fucus spiralis* macroalgae and influence of the extracts storage temperature—A short report☆

Lisete Paiva [a], Elisabete Lima [a,*], Ana Isabel Neto [b], José Baptista [a]

[a] *CITA-A—Research Center for Agricultural Technology, University of Azores, 9501-801 Ponta Delgada, S. Miguel, Azores, Portugal*
[b] *cE3c—Centre for Ecology, Evolution and Environmental Changes/Azorean Biodiversity Group, and University of Azores, Department of Biology, 9501-801 Ponta Delgada, S. Miguel, Azores, Portugal*

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ABSTRACT

Recently, increasing attention has been paid to the marine algae as a natural source of novel angiotensin-I converting enzyme (ACE) inhibitors, such as the phlorotannins that are the predominant polyphenols in brown algae. This study reports, for the first time, the ACE inhibition of methanol extract/fractions from Azorean brown algae *Fucus spiralis* (Fs) determined by HPLC-UV method, their total phenolic content (TPC) quantified as phloroglucinol equivalents (PE) and the effect of the Fs dry powder methanol extracts (Fs-DME) storage temperature on ACE inhibition. The results indicate that the ACE inhibition of Fs-DME decreased by 28.8% and 78.2% when stored during 15 days at −80 °C and −13 °C, respectively, as compared with the activity of Fs-DME at a refrigerated temperature of 6 °C and assayed immediately after extraction that showed a value of 80.1 ± 2.1%. This Fs-DME sample was fractionated by ultrafiltration membranes into three molecular weight ranges (<1 kDa, 1–3 kDa and >3 kDa), presenting the fraction >3 kDa remarkably high ACE inhibition (88.8 ± 2.4%), TPC value (156.6 ± 1.4 mg PE/g of dry weight fraction) and yield. Furthermore, chromatographic and spectrophotometric analyses corroborate that phenolic compounds were present in Fs methanol extract/fractions, and also revealed that phloroglucinol occurs in Fs. The results seem to suggest that Azorean Fs can be a source of powerful ACE-inhibitory phlorotannins with potential impact on public health, particularly on hypertensive patients.

1. Introduction

Macroalgae are living in highly competitive and aggressive surroundings, which are very different in many aspects from terrestrial environment. Such situations demand the production of quite specific and potent bioactive compounds which may lead to the development of novel drugs and/or functional foods or nutraceuticals [1]. Macroalgae have been consumed in Asian countries since ancient times and their dietary ingestion has been shown to decrease blood pressure in humans [2]. Angiotensin-I converting enzyme (ACE), a zinc metal protease, plays a key role in the control of blood pressure since catalyzes the conversion of angiotensin I to a potent vasoconstrictor angiotensin II and also promotes the degradation of the vasodilator bradykinin [3]. Therefore, the ACE inhibition has become a major target control for hypertension, a common progressive disorder leading to several chronic diseases such as cardiovascular disease, stroke, renal disease and diabetes [4]. In recent years, much attention has been paid to the potential of natural marine products as alternatives to synthetic drugs for the treatment of hypertension, due to their adverse side effects, [1,5] and some novel ACE-inhibitory compounds have already been isolated from algae species, such as the phlorotannins that are the predominant polyphenols found in brown algae [5,6]. These algae accumulate a variety of phlorotannins of low, intermediate and high molecular weights, ranging between 126 Da to 650 kDa, although they are more commonly found in the 10–100 kDa range.

Abbreviations: ACE, angiotensin I converting enzyme; DW, dry weight; Fs, *Fucus spiralis*; Fs-DME, Fs dry powder methanol extract; HA, hippuric acid; HHL, hippuryl-L-histidyl-L-leucine; PE, phloroglucinol equivalents; S/N, signal to noise; TPC, total phenolic content.

* Corresponding author.
E-mail address: elis@uac.pt (E. Lima).

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* Corresponding author.
E-mail address: elis@uac.pt (E. Lima).

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It is also known that the ACE-inhibitory activity may depend on the degree of polymerization of phlorotannin derivatives [5].

The Azores Islands, due to their location in the middle of the Atlantic Ocean associated with pristine seawaters, are a very promising location for marine natural resources that may produce new compounds with medicine-like effects in treating or preventing certain diseases. Traditionally, the Azorean population has gathered macroalgae either to eat or for chemicals extraction. The brown algae *Fucus spiralis* (Fs) is a local delicacy particularly the frond tips (the receptacles) that are picked and eaten fresh [7]. The present study aimed to: (1) determine the ACE-inhibitory activity of crude and size-fractionated methanolic extract from Fs using HPLC-UV method, (2) estimate their total phenolic content (TPC) expressed as phloroglucinol equivalents, (3) analyze them by chromatography (TLC and HPLC-DAD) and spectrophotometry (UV and IR) methodologies and (4) show the effect of the Fs dry powder methanol extracts (Fs-DME) storage temperature on ACE inhibition, in order to maximize its potential use in the preparation of antihypertensive drugs or functional foods.

2. Material and methods

2.1. Chemicals and reagents

Methanol (MeOH) and acetonitrile (ACN), HPLC grade, were purchased from Fluka Chemika (Steinheim, Switzerland). Chloroform ($CHCl_3$), etanol (EtOH), ethyl acetate (EtOAc), acetic acid, hydrochloric acid (HCl), orthophosphoric acid, sulphuric acid (H_2SO_4), sodium chloride, sodium hydroxide (NaOH), potassium bromide (KBr), trizma base, zinc chloride, hippuric acid (HA), hippuryl-L-histidyl-L-leucine (HHL), phloroglucinol (1,3,5-trihydroxybenzene), Folin-Ciocalteu reagent, vanillin and angiotensin I-converting enzyme (ACE) from porcine kidney were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrafiltration membrane system and membranes were purchased from Millipore Co (Bedford, MA, USA). Silica gel TLC plates were purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water purification and filtration system with 18 MΩ cm resistivity (Millipore, Bedford, MA, USA).

2.2. Collection, preparation, extraction and fractionation of F. spiralis (Fs) sample

The *F. spiralis* Linnaeus (Phaeophyceae, Ochrophyta) sample was collected in January 2013 from the littoral of São Miguel Island of Azores Archipelago (37° 40′ N and 25° 31′ W), Portugal, and a voucher specimen was prepared (voucher number SMG-13-04) and deposited in the Herbarium AZB – Ruy Telles Palhinha of the Department of Biology at the University of Azores. Within 24 h of collection, the Fs sample was first washed in seawater followed by distilled water to remove encrusting material, epiphytes and salts, and then air-dried and stored in an air-tight container in a freezer (–80 °C). Prior to the analytical procedures, the Fs sample was defrosted and dried at 40–45 °C for 48 h (avoiding overheating that could lead to oxidation), and then was grounded into a fine powder of 0.5 mm particle size, re-dried at 40 °C and stored in the dark under N_2 in a desiccator at a refrigerated temperature. The Fs-DME was prepared by suspending 1 g of dried algae powder in 100 mL solution of methanol:water (80:20 v/v) under continuous stirring at room temperature during 24 h. After centrifugation, the supernatant was dried under vacuum in a rotary evaporator at 40 °C and then dissolved in deionized water to adjust to 2 mg/mL concentration. The solution was then divided into three samples (ref. A, B and C), where the first one was used for the direct determination of ACE-inhibitory activity, immediately after extraction, and the other two were frozen at −80 °C and −13 °C, respectively, during 15 days for further determinations. The most active Fs-DME sample (ref. A) was further separated by ultrafiltration membranes with molecular weight cut-off of 1 and 3 kDa to obtain ultrafiltrates (Fr_{A1} <1 kDa, 1< Fr_{A2} <3 kDa and Fr_{A3} >3 kDa) that were then lyophilized.

2.3. ACE-inhibitory activity determination of Fs methanol extract/fractions

The determination of ACE-inhibitory activity was performed in vitro by RP-HPLC adapted from the spectrophotometric method described by Cushman and Cheung [8] with slight modifications. This method is based on the liberation of HA from HHL catalyzed by ACE. For the assay, 80 μL of the sample solution was pre-incubated at 37 °C for 5 min with 20 μL of ACE (2 mU/mL) enzyme. The mixture was subsequently incubated at the same temperature for 60 min with 200 μL of the substrate (5 mM HHL in 10 μM zinc chloride containing 100 mM sodium trizma base and 300 mM NaCl at pH 8.3). The reaction was terminated by adding 250 μL of 1 M HCl and the percentage of ACE inhibition was determined by an HPLC system. An aliquot of 10 μL from the reaction mixture was analyzed on a reverse-phase Ultrasphere C_{18} column (250 × 4.6 mm i.d., 5 μm particle size) (Beckman Coulter, Miami, FL, USA) using an isocratic elution of MeOH:ACN:0.1% HCl (25:25:50 v/v/v) at a constant flow-rate of 0.5 mL/min and HA and HHL were detected by UV at 228 nm. The percentage of ACE inhibition was calculated as follows:

$$\text{ACE inhibition}\,(\%) = \frac{B - A}{B - C} \times 100$$

where A is the absorbance of HA generated in the presence of ACE inhibitor, B the absorbance of HA generated without ACE inhibitor and C the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay). Captopril was used as a positive control for ACE inhibition. The repeatability of the HA HPLC analysis was evaluated in intraday and interday measurements of the retention time by repeated injections (n = 5). The recovery was calculated based on the difference between the total concentration determined in the spiked samples and the concentration observed in the non-spiked samples. The linearity of HA was evaluated by the calibration curve constructed in triplicate with five different concentrations.

2.4. Total phenolic content (TPC) determination of Fs methanol extract/fractions

The TPC was determined according to the Folin–Ciocalteu method as previously described [9], using phloroglucinol (a basic structural unit of phlorotannins) as a standard and expressing the results as mg of phloroglucinol equivalents (PE) per g of dry weight (DW) sample.

2.5. Chromatographic and spectrophotometric analysis of Fs methanol extract/fractions

The thin-layer chromatography (TLC) was performed on a silica gel plate (Kieselgel 60F, 0.25 mm). An aliquot of each Fs sample and phloroglucinol standard was spotted on the silica gel plate with a solvent system of $CHCl_3$:MeOH:water:acetic acid (65:25:4:3, v/v). The spots were visualized by spraying the plates with vanillin-H_2SO_4 solution. Red color spots produced indicate the presence of phenolic compounds [10]. The HPLC-DAD analysis of Fs-DME was performed using the following analytical conditions: column Prevail C_{18} (250 × 4.6 mm i.d, 5 μm particle size) from the Grace (The Nest Group, Southborough, MS, USA), mobile phase (A) 89.5% orthophosphoric acid 0.1% + 2% EtOAc + 8.5% ACN and phase (B) ACN:H_2O (1:1 v/v), gradient elution (t = 0–10 min, 0% B and

t = 20 min, 60% B) and flow-rate of 0.6 mL/min. Spectra data were sweeped from 220 to 400 nm, and the chromatogram was recorded at 280 nm. Phloroglucinol was identified by retention time based on comparison with the authentic standard and by spiking the sample with standard. The UV spectra (in EtOH) of Fs fractions were recorded on a Shimadzu model 1800 UV/VIS spectrophotometer. The bathochromic shift of the absorption maxima was determined by adding 2 drops of a 2 M sodium hydroxide solution to the cuvette sample solutions [11]. The infrared spectra (500–4000 cm^{-1}) of Fs fractions were recorded in KBr disks with a Fourier transform IR spectrophotometer (Perkin Elmer model Frontier IR). One milligram of dry sample was mixed with 100 mg of dry KBr, and the mixture was pressed into a disk [11].

2.6. Statistical analysis

All data were expressed as mean ± standard deviation (SD) of three determinations. Statistical comparison was performed by using one-way analysis of variance (ANOVA). Differences between means at the 5% ($p < 0.05$) level were considered significant.

3. Results and discussion

3.1. HPLC ACE-inhibitory activity assay

The presented HPLC method completely eliminates the interference from HHL during the quantification of the HA as compared with spectrophotometric method of Cushman and Cheung [8]. Complete baseline separation of HA and HHL was achieved in less than 10 min under the analytical conditions described in methods. The detector response for pure HA samples was found to be linear over the range of 0.10 μg to 4.01 μg/mL. The linear regression analysis provided the following equation:

$$Y = (2 \cdot 10^7)x + 4 \cdot 10^6,\ R^2 = 0.997$$

The results show that the intraday repeatability was 1.72%, whereas the interday precision (data acquired over a period of 5 days) was better than 2.85% indicating a high degree of the repeatability. The recovery was calculated as referred in methods. The relative standard deviation was better than 2.35% and the mean recovery ranged from 97.7% to 99.1% indicating a high degree of the method accuracy for the determination of HA under the analytical conditions used. The limit of HA detection, defined as the sample amount injected which gave a signal to noise (S/N) ratio of 3, was determined to be 0.1 μg/20 μL injected, and the limit of quantification (S/N ratio of 10:1) was 0.5 μg/20 μL injected. In order to further confirm that HA is a product of ACE-catalyzed hydrolysis of HHL the reaction mixture was acidified with 1 M HCl before the addition of ACE. Complete inhibition of ACE reaction was obtained in the acidic conditions and no HA was detected (results not shown). Therefore, the HPLC provides a simple, rapid and accurate method for the ACE-inhibitory activity assay.

3.2. ACE-inhibitory activity, TPC, and chromatographic and spectrophotometric analysis of Fs methanol extract/fractions

The search for ACE-inhibitors from natural resources, such as marine organisms including macroalgae, has become one of the major areas of research in the field of nutraceutical and pharmaceutical industries [5]. Within this context, the present study on the ACE inhibition of methanolic extract from Fs showed a strong activity with a value of 80.1% (see Fig. 1, sample A). This extract presented also high recovery yield (213.0 mg/g DW algae) and TPC value (212.7 mg PE/g DW extract), as shown in Table 1. These results indicated the efficiency of the extraction process used, and also the economic viability of this crude extract commercialization as an ingredient for antihypertensive functional foods and nutraceuticals formulation. In order to enhance the ACE inhibition, the extract was further separated by membranes with molecular weight cut-off of 1 and 3 kDa. The ACE inhibition percentage and the TPC of the three fractions obtained (Table 1) showed significant differences, ranging from 7.2 to 88.8% and 9.4–156.6 mg PE/g DW, respectively, the higher values being recorded in fraction >3 kDa (Fr_{A3}) probably as a result of the higher concentration of potent active compounds. The recovery yields from the ultrafiltration membrane fractions were also reported in Table 1, presenting the Fr_{A3} the highest value. It should be pointed out that in the ultrafiltration process some amount of the starting sample can be lost on the filters, as observed in the results presented in Table 1.

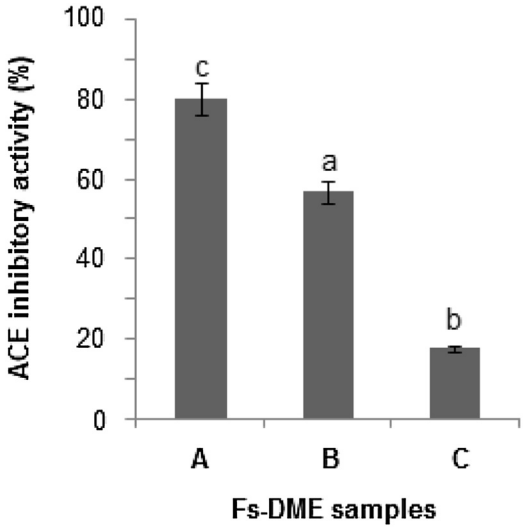

Fig. 1. Comparison of angiotensin-I converting enzyme (ACE) inhibitory activities of *Fucus spiralis* dry powder methanol extract (Fs-DME) sample stored at different temperatures: refrigerated temperature of 6 °C and assayed immediately after the extraction (sample A) and frozen during 15 days at −80 °C (sample B) and −13 °C (sample C). Values are mean ± SD (n = 3). Different letter are significantly different ($p < 0.05$). Tested concentration: 200 μg/mL.

Previous studies on the ACE inhibition of methanolic or ethanolic extracts from some other brown algae indicated also a strong activity [e.g. 6,12]. A significant increase in TPC was also observed in the high-molecular-weight fraction (> 3.5 kDa) of the ethanolic extracts from Irish Fs and other Fucaceae macroalgae that are reported to be rich sources of phlorotannins (brown-algal polyphenols) [13]. However, relatively limited characterization of phlorotannins has been carried out. This is possibly the consequence of their structural complexity due to their polymeric nature. The Fs-DME and its ultra-filtrate fractions were further analyzed by chromatography and spectrophotometry methodologies. In the HPLC-DAD of Fs-DME the results revealed the presence of phenolic compounds since they are characterized by their UV absorption maximum at approx. 270 nm with a shoulder at 285 nm, deduced from DAD detection, as previously reported [14]. In the TLC chromatogram, red spots were detected in all samples. UV spectral data of Fs ultra-filtrate fractions suggested that the absorption maxima had a bathochromatic shift (16 nm) with NaOH addition. The IR spectra of these fractions revealed the existence of a hydroxyl group (3415 cm^{-1}) and an aromatic ring (1260, 1620, 2970, and 2850–2970 cm^{-1}), indicating the presence of polyphenols with phloroglucinol unit [11]. As a result, all the referred analyses corroborate that phenolic compounds (phlorotannins) were present in Fs methanol extract/fractions. Additionally, HPLC-DAD and TLC

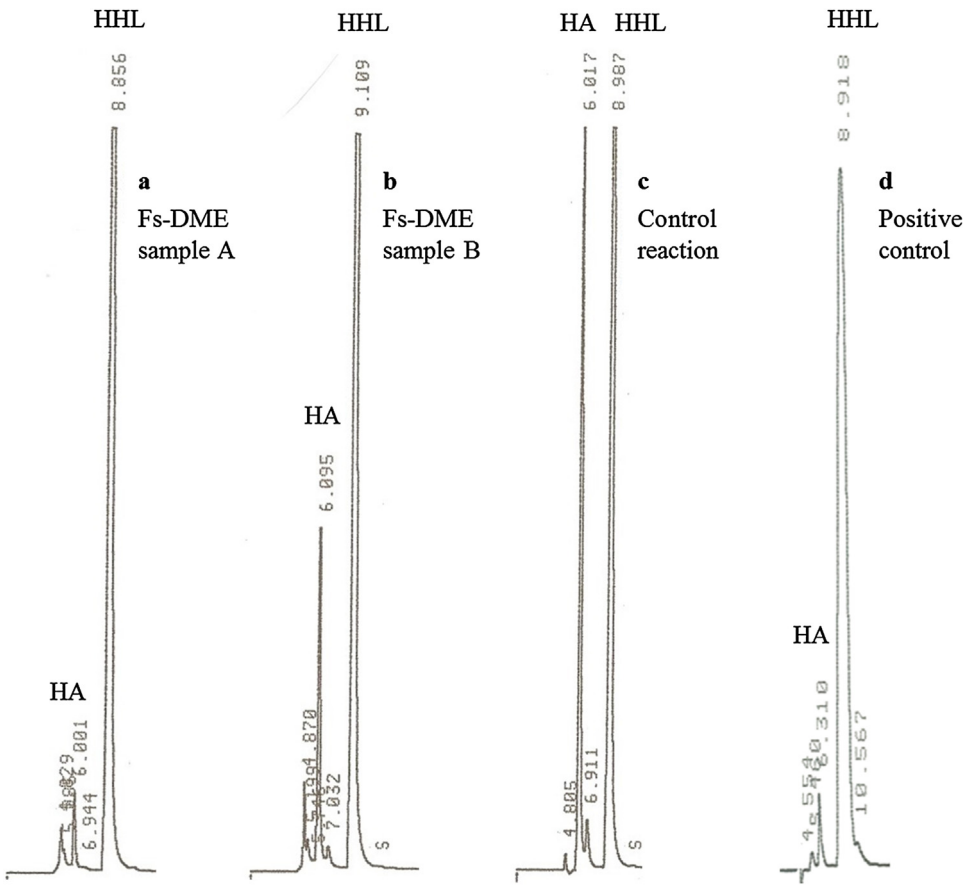

Fig. 2. Representative HPLC chromatograms of angiotensin-I converting enzyme (ACE) reaction mixture using the inhibitory *Fucus spiralis* dry powder methanol extract (Fs-DME) sample stored at different temperatures: refrigerated at 6 °C and assayed immediately after the extraction (a) and frozen at −80 °C during 15 days (b), as compared to control sample (HHL plus ACE without Fs-DME) (c) and positive control (d) chromatograms. Analytical chromatographic conditions as referred in methods. Legend: HA = hippuric acid and HHL = hippuryl-L-histidyl-L-leucine.

Table 1
Angiotensin-I converting enzyme (ACE) inhibitory activity, recovery yield and total phenolic content (TPC) of the fractions obtained by ultrafiltration from the Fs-DME (*Fucus spiralis* dry powder methanol extract) sample storage at a refrigerated temperature of 6 °C and immediately assayed after extraction (tested concentration 200 μg/mL).

Ref.	Sample	Recovery yield (mg/g DW algae)	ACE inhibition (%)[a]	TPC (mg PE/g DW sample)
A	Unfractionated Fs-DME	213.0	80.1 ± 2.1^{c}	212.7 ± 2.3^{c}
A1	$Fr_1 < 1000$ Da	27.8	17.3 ± 0.5^{b}	23.3 ± 0.5^{a}
A2	$1000 < Fr_2 < 3000$ Da	11.5	7.2 ± 0.2^{b}	9.4 ± 0.3^{a}
A3	$Fr_3 > 3000$ Da	143.2	88.8 ± 2.4^{c}	156.6 ± 1.4^{b}

Values are mean ± SD (n = 3). Different superscript letters are significantly different ($p < 0.05$). DW = dry weight. PE = phloroglucinol equivalents.
[a] Captopril showed an ACE inhibition value of 97.8 ± 1.8% (at 200 μg/mL concentration).

analysis revealed also the presence of the monomer phloroglucinol in Fs-DME. Phloroglucinol was previously isolated from Japanese Laminariaceae, another phlorotannin-rich brown algae family [10].

According to some studies, brown algae might have strong ACE-like inhibitors associated not only with peptides but also with phlorotannins [5,6]. Wijesinghe et al. [6] reported that the ACE inhibition may be closely associated with protein-binding abilities of phlorotannins. It has been well described that tannins have the ability to form strong complexes with proteins, either reversibly by hydrogen bonding through peptide or amide linkages or irreversibly by covalent condensation. Thus, the obtained evidence in the previous [6,13] and present studies suggest that the ACE inhibition of Azorean Fs may be due to its rich content of high-molecular-weight phlorotannins. However, further analysis will be performed in future work to provide structural information of the ACE-inhibitory compounds from Fs.

3.3. Effect of the Fs-DME storage temperature on ACE inhibition

The results concerning the ACE inhibition by the investigated Fs-DME samples (see Fig. 1) showed that the strongest inhibitory activity was from sample A (kept dry at a refrigerated temperature of 6 °C and assayed immediately after extraction) followed the sample B (kept frozen at −80 °C during 15 days before biological assay) with 80.1 ± 2.1% and 57.0 ± 1.5% of ACE inhibition ($p < 0.05$), respectively. The lowest inhibitory activity was recorded for sample C (kept frozen at −13 °C during 15 days before biological assay) namely 17.5 ± 0.5% of ACE inhibition ($p < 0.05$). The representative chromatograms of ACE reaction mixtures (Fig. 2a, b) show that sample A have lower liberation of HA as compared with sample B. The Fig. 2c serves as control reaction (HHL plus ACE without sample) and Fig. 2d as a positive control for ACE inhibition. The results indicate that the ACE-inhibitory activity of Fs-DME decreased by

28.8% and 78.2% when stored during 15 days at −80 °C and −13 °C, respectively, as compared with the activity of Fs-DME kept dry at refrigerated temperature of 6 °C and assayed immediately after extraction. Based on this evidence, some of the methanol extract components are not stable during storage even at low temperatures of −80 °C.

4. Conclusion

The literature on the ACE inhibition from species belongings to *Fucus* genus is sparce. This study indicates that the high-molecular-weight fraction (>3 kDa) from Fs-DME presented significantly higher ACE inhibition, TPC and yield, than the other fractions. TLC and UV and IR spectra analysis confirm indeed that phenolic compounds (phlorotannins) were present in this Fs fraction. The present findings seem to suggest that the Azorean Fs macroalgae can be a potential source of powerful ACE-inhibitory phlorotannins that may be used for the development of nutraceuticals and pharmaceuticals to prevent and/or to treat hypertension that would be economical, safer and a natural alternative therapy to commercial synthetic drugs. Additionally the study shows the best extract storage conditions to keep the ACE-inhibitory activity. The results clearly indicate that ACE inhibition of Fs-DME decreased when the samples are stored at frozen temperature, even at −80 °C, as compared with the activity of samples at a refrigerated temperature and assayed immediately after extraction. Therefore, the ACE inhibition assay must be performed on the algae methanol extracts of the refrigerated dry powder kept under a N_2 atmosphere in order to avoid the decrease of the inhibitory activity.

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