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Original Article

Evaluation of acetylcholinesterase inhibitory activity of Brazilian red macroalgae organic extracts

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ABSTRACT

Alzheimer's disease affects nearly 36.5 million people worldwide, and acetylcholinesterase inhibition is currently considered the main therapeutic strategy against it. Seaweed biodiversity in Brazil represents one of the most important sources of biologically active compounds for applications in phytotherapy. Accordingly, this study aimed to carry out a quantitative and qualitative assessment of *Hypnea musciformis* (Wulfen) J.V. Lamouroux, *Ochtodes secundiramea* (Montagne) M.A. Howe, and *Pterocladiella capillacea* (S.G. Gmelin) Santelices & Hommersand (Rhodophyta) in order to determine the AChE effects from their extracts. As a matter of fact, the *O. secundiramea* extract showed 48% acetylcholinesterase inhibition at 400 μg/ml. The chemical composition of the bioactive fraction was determined by gas chromatography–mass spectrometry (GC–MS); this fraction is solely composed of halogenated monoterpenes, therefore allowing assignment of acetylcholinesterase inhibition activity to them.

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder characterized by a loss of basal forebrain cholinergic neurons and reduced level of the neurotransmitter acetylcholine (ACh) (Alzheimer's Association, 2014). AD affects up to 5% of people over 65 years of age, rising to 20% of those aged over 80 years, suggesting the importance of treating this disease based upon increased life expectancy, particularly in developed countries worldwide. Specifically, estimates show that 27.7 up to 35 million people were affected by this disease from 2005 through 2010, respectively. In this same survey, it was found that treatment costs increased from 156 USD billion to 604 billion (Wimoa et al., 2007, 2013).

Control over AD is achieved by extending the action of acetylcholine (ACh) via acetylcholinesterase inhibition (AChEI). Current drugs exhibit two action mechanisms, either prosthetic or acid-transferring. Prosthetic inhibitors have an affinity for the anionic site of acetylcholinesterase and prevent acetylcholine from accessing it (competitive inhibitors). Acid-transferring inhibitors react with the enzyme and form an intermediate compound. Depending on the stability of this product, the effects could be short-term and reversible or long-acting and irreversible (Nair and Hunter, 2004).

The current drugs used against AD are plant-derived alkaloids: rivastigmine and galantamine. These drugs are AChEIs and can be used to treat early and moderate stages of AD by increasing the endogenous levels of acetylcholine to boost cholinergic neurotransmission (McGleenon et al., 1999). Although these drugs display some undesirable side effects, such as hepatotoxicity and gastrointestinal disorder, there is a growing interest in finding new AChE inhibitors from natural sources, but with few off-target effects (Rhee et al., 1997).

Marine organisms have a high chemical diversity of ecologically active substances with protective functions against predators, biofoulers and epiphytes, as well as against constant changes in abiotic conditions tolerated by algae in the marine coastal environment (Amsler, 2008; Ferreira et al., 2012; Mesko et al., 2015). Such diversity provides a useful resource for the discovery of novel bioactive carbon compounds, many of them with potential applications in the pharmaceutical, nutraceutical, and agricultural fields (Smit, 2004; Cardozo et al., 2006; Gressler et al., 2011a, 2011b; Torres et al., 2014).

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Seaweed extracts have been tested against ACh enzyme with successful results (Natarajan et al., 2009). As such, seaweeds represent a promising group of species for developing new marine natural products based on the ease of obtaining biomass by performing cultures for the exploitation of bioactive compounds (Baweja et al., 2009 and therefore contributing to the conservation of natural populations (Yokoya and Yoneshigue-Valentin, 2011).

Hence, motivated by the economic and social impact of AD, this study was aimed at testing the qualitative and quantitative AChEI activity of organic extracts obtained from three Brazilian red algae. The chemical composition of the bioactive fractions was investigated by gas chromatography–mass spectrometry (GC–MS).

Materials and methods

Algal material and extraction

Two hundred grams (fresh weight; equivalent to thirty individuals of each species) of *Hypnea musciformis* (Wulfen) in J.V. Lamouroux, *Ochtodes secundiramea* (Montagne) M.A. Howe and *Pterocladiella capillacea* (S.G. Gmelin) Santelices & Hommersand were collected from a lateritic reef at Manguinhos Beach (20°11′12″ S, 40°11′24″ W) in the municipality of Serra, Espírito Santo State, Brazil. After collection, seaweed biomass was cleaned and immediately transported to the laboratory in dark plastic flasks containing filtered seawater maintained at 22 °C by means of thermal packaging. Furthermore, the seaweeds were properly identified and deposited in the Herbarium VIES, Universidade Federal do Espírito Santo, under voucher numbers 18.853, 18.854 and 18.855, corresponding to *H. musciformis*, *O. secundiramea*, and *P. capillacea*, respectively.

The freshly cleaned seaweeds were weighed and then immediately extracted by using 10 ml of dichloromethane/methanol (DCM/MeOH) (2:1, v/v) per 1 g of seaweed (Machado et al., 2014a). The material was maintained in a dark room at 20 °C for one week, and then the extracts were filtered through a Whatman No. 5 filter paper and concentrated by under low pressure. The extraction yield was calculated for each algal sample. All chemicals used were of analytical grade (Merck, Darmstadt, Germany).

Inhibitory activity of acetylcholinesterase (AChEI)

Qualitative evaluation by autographic assay

The AChEI activity of DCM/MeOH crude extract was detected by using a thin-layer chromatography (TLC) autographic assay as previously described (Marston et al., 2002). Aliquots of 100 μg of each dried seaweed extract and 0.3 μg of physostigmine (Sigma, used as positive control) were dissolved, spotted on TLC layers (Silica gel 60 F254, 10 × 10 cm, layer thickness 0.2 mm, E. Merck, Germany), which were developed with mobile phase hexane:ethyl acetate:methanol (2:7:1 v/v/v), and then dried. Next, the plates were sprayed with the enzyme solution (6.66 U/ml) (Electric eel AChE type V, product no. C 2888, 1000 U – Sigma–Aldrich), thoroughly dried, and incubated in a humid atmosphere at 37 °C for 20 min. Afterwards, the plates were sprayed with 0.25% of 1-naphthylacetate in ethanol (5 ml) plus 0.25% of aqueous Fast Blue B salt solution (20 ml). The spots corresponding to potential acetylcholinesterase inhibitors were identified as clear zones against a purple background.

Retention factor values (R_f) of bioactive compounds were determined and employed for their preparative scale isolation by thin-layer chromatography (20 cm × 20 cm, layer thickness 1.5 mm, 60 F 254, Sigma–Aldrich). Extract samples of 80 mg were applied on preparative plates which were developed under identical conditions to those used at analytical scale essays. After elution, the AChE-positive regions were removed from the plates and extracted with DCM/MeOH (90:10 v/v).

Quantitative evaluation by microplate assay

AChE activity was measured using a modified 96-well microplate assay based on Ellman's method (Ellman et al., 1961, as modified by Rhee et al., 2001). Such extremely sensitive method is based on measuring thiocholine production when acetylthiocholine is hydrolyzed. This is accomplished by the continuous reaction of thiol with 5,5′-dithiobis(2-nitrobenzoic acid). Solutions: A. Tris/HCl 50 mM, pH 8; B. Tris/HCl 50 mM, pH 8, with 0.1% bovine albumin fraction V; C. Tris/HCl 50 mM, pH 8, with NaCl (0.1 M) and $MgCl_2 \cdot 6H_2O$ (0.02 M).

To each well of a 96-well microplate, 25 μl acetylthiocholine iodide (15 μM), 125 μl of 5,5′-dithiobis (2-nitrobenzoic acid) in solution C (3 μM DTNB or Ellman's reagent), 50 μl of solution B, 25 μl of seaweed extract dissolved in MeOH and diluted in solution A at concentrations of 1.56, 12.5, 25, 50, 100, 200 and 400 μg/ml were added. The absorbance was measured at 405 nm for 30 sec. After 25 μl of the enzyme AChE (0.22 U/ml) was added, the absorbance was again read every 5 min of incubation for four times. The percentage of AChE inhibition was calculated by comparing the reaction rates of samples to the negative control (10% MeOH in solution A, considering 100% as the total activity of AChE). Physostigmine was used as standard at concentrations ranging from 1.56 to 400 μg/ml. A BioTek ELISA reader (PowerWave™ HT Microplate Reader and KC4™ software) was used to determine reaction rates. This analysis was carried out in triplicate ($n = 3$) to calculate the mean and standard deviation.

CG–MS analysis of bioactive fraction obtained from O. secundiramea extract

Currently, different strategies have been reported in the literature in order to estimate algal compounds applying CGMS (Gressler et al., 2009). The bioactive fraction obtained from *O. secundiramea* was analyzed by a gas chromatograph-mass spectrometer (GCMS-QP2010 Plus, Shimadzu, Japan) provided with a HP-5MS column (30 m × 0.25 mm × 0.1 μm). The samples were injected in split mode at 220 °C, and the transfer line was set to 240 °C. Helium (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/min. A linear oven temperature program was employed. The temperature was ramped from 60 to 260 °C at a rate of 3 °C/min and then held at 260 °C for 40 min. Detection was performed in full scan mode, mass-to-charge ratio (m/z) 50–650. Electron impact ionization was employed (collision energy = 70 eV), and the mass spectrometer ion source was maintained at 240 °C. Compounds were identified by their GC retention times and mass spectra (NIST08 Mass Spectral Library and literature data). The area of each peak in the total ion chromatogram was integrated.

Results and discussion

Fig. 1 shows the qualitative results of the autographic assay of acetylcholinesterase inhibition activity (AChEI), and Table 1 displays the fresh seaweed biomass weights, dried extract and extraction yield along with AChEI data of the autographic assay: R_f and bioactive compound intensity.

The AChEI activity of plant extracts is classified according to Vinutha et al. (2007) as potent inhibitors (greater than 50% inhibition), moderate inhibitors (30–50% inhibition) and weak inhibitors (below 30% inhibition). According to this classification, the extracts of *O. secundiramea* have moderate activity (48.59 ± 0.8%), while *H. musciformis* and *P. capillacea* extracts have weak activity (7.21 and 5.38%, respectively), at a higher concentration (Fig. 2).

As described in the literature, seaweed extracts exhibit poor anti-AChE activity, with IC_{50} ranging from 2.6 to 10 mg/ml, far