A comparative evaluation of biological activities and bioactive compounds of the seagrasses *Zostera marina* and *Z. noltei* from Southern Portugal

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Abstract

This work assessed the antioxidant potential, acetylcholinesterase (AChE) inhibition and the *in vitro* cytotoxic activity of extracts of the seagrasses *Zostera marina* and *Z. noltei* collected from southern Portugal. The total phenolic contents (TPC), the rosmarinic acid (RA) concentration (HPLC/DAD) and the fatty acid profile (GC/MS) is also described. *Z. marina* had the highest TPC, radical scavenging activity against DPPH radicals, and copper chelating activity. *Z. noltei* had metal chelation capacity to copper and iron ions. None of the species was able to inhibit AChE. Both seagrasses had high levels of polyunsaturated fatty acids. *Z. marina* significantly and selectively reduced the viability of tumorous neuronal cells. *Z. noltei* was highly toxic for the three cell lines tested and was selective against hepatocarcinoma cells at the concentration of 100 µg/mL. RA was the main compound identified in *Z. marina*, but not in *Z. noltei*.

Keywords: Cytotoxicity; lipids; oxidative stress; PUFA; rosmarinic acid, seagrasses

1. Experimental

1.1. *Chemicals*

All chemicals used in the experiments were of analytical grade. acetylcholinesterase (AChE) (EC.3.1.1.7) from electrical eel, acetylthiocholine iodide (ATChI), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), galanthamine, pyrocatechol violet (PV), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Supelco® 37 component fatty acids methyl esters (FAME) mix were purchased from Sigma (Steinheim, Germany). Ethylenediamine tetraacetic acid (EDTA) and sodium carbonate were from Fluka (Steinheim, Germany). Merck (Darmstadt, Germany) supplied ferrozine, copper sulphate pentahydrate and Folin-Ciocalteu (F-C) reagent, while methanol was from Fischer Scientific (Loughborough, UK). Additional reagents and solvents were purchased from VWR International (Leuven, Belgium).

1.2. *Sample collection*

Biomass from *Z. marina* was collected from meadows on Culatra Island (Portugal), while that from *Z. noltei* was sampled from the intertidal zone of the Ria Formosa lagoon (Portugal), in the winter of 2011 / 2012. Samples were identified by Dr. Aschwin H. Engelen, and voucher specimens are kept at the MarBiotech laboratory under codes MBS01 (*Z. noltei*) and MBS02 (*Z. marina*). Samples were washed with freshwater, freeze-dried, grounded and stored in the dark at -20 °C until use.

1.3. *Preparation of the extracts and extraction yield*

The extracts were prepared according to the method described by Achamlale et al. (2009), with some modifications. Dried biomass was mixed with 70% aqueous methanol (1:40, w/v) and extracted overnight under stirring at room temperature (RT). Extracts were filtered (Whatman No. 4), evaporated to dryness at 50°C in a rotary evaporator under reduced pressure (BUCHI R-210, Flawil, Switzerland), and the obtained dry extract was weighed in order to determine the extraction yield (%). Then, the dried methanol extract was resuspended in water and defatted with chloroform in a separating funnel. The resulting solution was extracted three times with ethyl acetate, the fractions were combined, and after water removal by the addition of anhydrous sodium sulphate they were evaporated to dryness in a rotary evaporator and weighed. Dried fractions were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 25 mg/mL and stored in the dark at 4 °C.

1.4. *Phytochemical characterization of the extracts*

1.4.1. Determination of total phenolics content (TPC)

TPC was determined on extracts at the concentration of 10 mg/mL by the F-C method (Julkunen-Tiitto, 1985) as described previously (Custódio et al., 2014). Briefly, samples (5 µL) were mixed with 10-fold diluted F-C reagent in distilled water (100 µL) and incubated at RT for 5 min. Then, 100 µL of sodium carbonate (75 g/L, w/v) were added and the absorbance was measured at 725 nm after a 90 minutes incubation period at RT, on a microplate reader (Biotek Synergy 4, Biotek instruments, Winooski, USA). TPC was expressed as gallic acid equivalents (GAE) in milligrams per gram of dried extract (dry weight, DW), using a calibration curve made of GA at six different concentrations (0-1.5 mg/mL).

1.4.2. High-performance liquid chromatography (HPLC) analysis

Samples (20 µL) were analyzed by HPLC consisting of a liquid chromatography system (Waters 2795 PDA 2996 ZQ Micromass 4000), containing a vacuum degasser, quaternary pump, autosampler, thermostated column compartment and variable wavelength detector. Separations were carried out at 30°C on a Luna C18 reverse phase analytical column (Phenomenex, Torrance, CA) 100 Å pore size, 250 × 4.6 mm, 5 µM particle size. Linear gradients of acetonitrile (0.036% trifluoroacetic acid) into distilled water (0.045% trifluoroacetic acid) were run at a flow rate of 1.0 mL/min over 45 min. UV detection was performed at a range of 210-400 nm. Data were managed with Masslynx software. Peak identification was accomplished by comparing retention times and UV spectra with those of the authentic standard of RA (purity, 98%). Quantitative determinations were carried out by peak area measurements at 330 nm, using a calibration curve of RA at the same wavelength.

1.5. *Analysis of the fatty acids methyl esters (FAME) profile by GC/MS*

The FA were extracted, converted to the corresponding FAME, and analyzed by gas chromatography–mass spectrometry (GC/MS) as described previously (Custódio et al., 2014). "Supelco® 37 component FAME mix" (Sigma-Aldrich, Steinheim, Germany), was used to produce the required calibration curves for the identification and quantification of the FAME.

1.6. *Antioxidant activity*

1.6.1. DPPH free radical scavenging activity (RSA)

The RSA against DPPH was determined by the method described by Custódio et al. (2014) on extracts at concentrations ranging from 0.125 to 10 mg/mL. Samples (22 µL) were mixed with 200 µL of a methanol DPPH solution (120 µM) in 96-well flat bottom microtitration plates, and incubated in darkness at RT for 30 min. The absorbance was measured at 515 nm (Biotek Synergy 4, Biotek instruments, Winooski, USA) and results were expressed as inhibition (%) relative to a control containing DMSO. Butylated hydroxytoluene (BHT, 1 mg/mL) was used as a positive control.

1.6.2. Metal chelating activity on copper (MCAC)

MCAC was determined according to Megías et al. (2009). Briefly, samples (30 µL at concentrations ranging from 0.125 to 10 mg/mL) were mixed with 200 µL of 50 mM Na acetate buffer (pH 6), 6 µL of 4 mM PV in the same buffer and 100 µL of copper (II) sulfate pentahydrate in 96-well microplates The change in colour was measured at 632 nm using a microplate reader (Biotek Synergy 4, Biotek instruments, Winooski, USA). Results were expressed as inhibition (%) relative to a control containing DMSO. The synthetic metal chelator EDTA was used as a positive control at the concentration of 1 mg/mL.

1.6.3. Metal chelating activity on iron (MCAI)

MCAI was determined by measuring the formation of the Fe^{2+}-ferrozine complex according to Megías et al. (2009), with some modifications. Samples (30 µL at concentrations ranging from 0.125 to 10 mg/mL) were mixed in 96-well microplates with 200 µL of distilled water and 30 µL of an iron (II) chloride solution (0.1 mg/mL in water), and incubated for 30 min. at RT. Then, 12.5 µL of ferrozine solution (40 mM in water) was added, and change in colour was measured in a microplate reader at 562 nm (Biotek Synergy 4, Biotek instruments, Winooski, USA). EDTA was used as a positive control at the concentration of 1 mg/mL, and results were expressed as inhibition (%) relative to a control containing DMSO.

1.7. AChE inhibition assay

The AChE inhibitory activity was determined using the method described by Orhan et al. (2007). Briefly, 140 µL of 0.1 mM sodium phosphate buffer (pH 8.0) was mixed with 20 µL of the extracts at different concentrations (0.125 to 10 mg/mL) and 20 µL of AChE (0.28 U/mL) in 96-well microplates, and incubated for 15 min. at 37ºC. Then, 10 µL of ATChI (4 mg/mL) were added, together with 20 µL of DTNB (1.2 mg/mL). Absorbances were read 10 min. later at 412 nm, in a 96-well microplate reader (Biotek Synergy 4). Results were expressed as enzymatic activity (%) relative to a control containing DMSO. Galanthamine (1 mg/mL) was used as a positive control.

1.8. In vitro *cytotoxic activity*

1.8.1. Cell lines and culture conditions

HepG2 (human hepatocellular carcinoma) cells were maintained in RPMI-1640 culture media supplemented with glucose (1 g/mL), 10% FBS (v/v), L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL). S17 (bone marrow stromal) cell line was grown in DMEM culture medium supplemented with glucose (1g/mL), 10% FBS, L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL). SH-SY5Y (human neuroblastoma) cells were grown in 1:1 mixture of EMEM and Ham's F12 Nutrient-Mixture supplemented with 10% FBS and 1% penicillin. All cell lines were grown in an incubator at 37ºC and 5.0% CO_2 in humidified atmosphere.

1.8.2. Determination of cell viability

For the determination of the effect of the extracts on cell viability exponentially growing cells were seeded at a density of 5×10^3 cells / well on 96-well plates and incubated for 24h at 37ºC in 5% CO_2. The extracts were then applied for 72h at different concentrations (3-100 µg/mL). Negative control cells were treated with DMSO at the highest concentration used in test wells (0.5%, v/v), and cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983) as described previously (Custódio et al., 2014). Absorbance was measured at 590 nm, and results were expressed in terms of cell viability (%) and as IC_{50} values (µg/mL). The selectivity index (SI) was estimated using the following equation: SI = CT/CNT, where CT and CNT indicate the extract-induced cytotoxicity on tumoural cells (HepG2, SH-SY5Y) and on non-tumoural cells (S17), respectively. A SI >1 indicates selective toxicity on tumour cells.

1.9. *Statistical Analyses*

Results were expressed as mean ± standard deviation (SD), of at least three replicates. Significant differences were assessed by analysis of variance (ANOVA) or using Kruskal-Wallis test ($p < 0.05$) when parametricity of data did not prevail (SPSS statistical package for Windows, release 15.0). The IC_{50} values were calculated with GraphPad Prism V 5.0.

2. References

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Figures

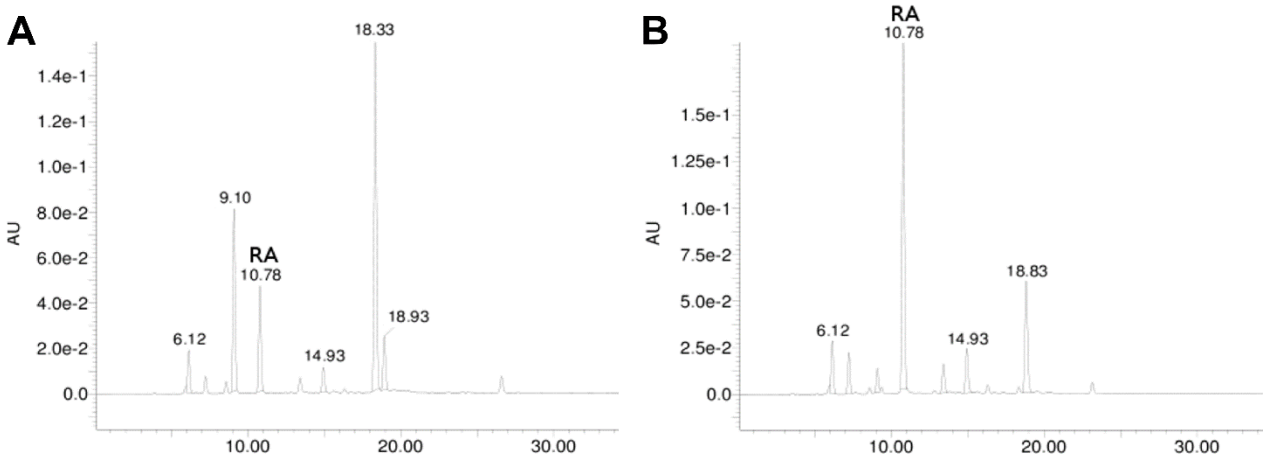

Fig. S1. Analysis of ethyl acetate fractions of crude methanol extracts of *Z. noltei* (A) and *Z. marina* (B) by HPLC (the effluent was monitored at 330nm). RA indicates the rosmarinic acid peak.

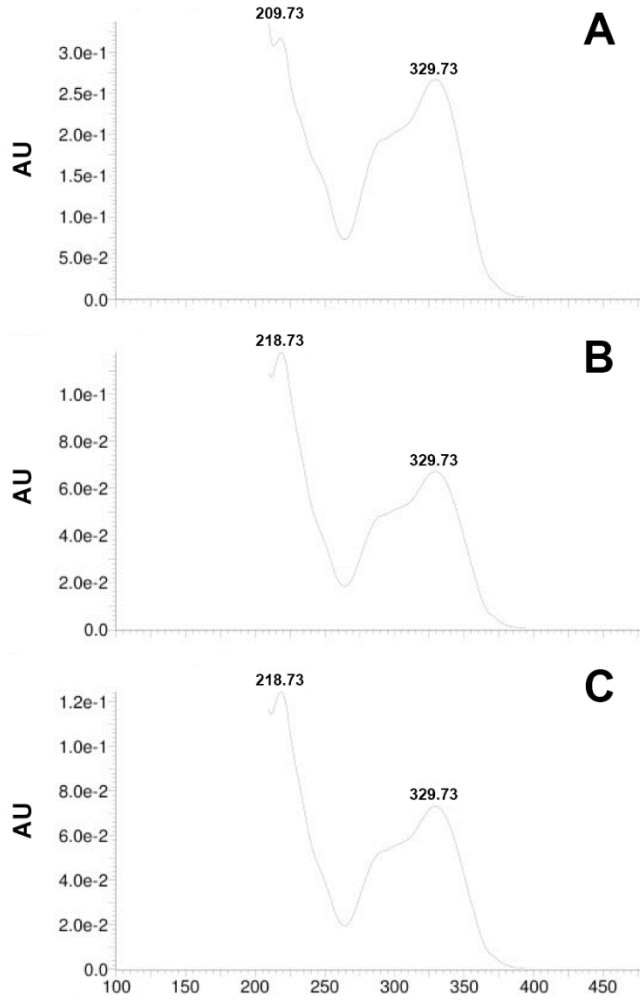

Fig. S2. UV spectra of the rosmarinic acid peak from *Z. marina* (A), *Z. noltei* (B) and RA standard (C).