SUPPLEMENTARY MATERIAL

First report of the nutritional profile and antioxidant potential of *Holothuria arguinensis*, a new resource for aquaculture in Europe

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Abstract

This work reports for the first time the nutritional profile and antioxidant potential of the edible sea cucumber *H. arguinensis* from the Northeastern Atlantic. *H. arguinensis* has high levels of protein, with the amino acids profile dominated by alanine, glycine and proline and low lysine/arginine ratios. Its carbohydrate and energetic contents are also low as well as the total lipid levels, although its lipid profile is rich in polyunsaturated fatty acids (PUFA), especially arachidonic, eicosapentaenoic and docosahexaenoic acids. In addition, *H. arguinensis* has high levels of calcium. The water and ethanol extracts show ability to scavenge free radicals and to chelate copper and iron ions. Our results indicate that *H. arguinensis* has a balanced nutritional quality suitable for human consumption. Besides it contains compounds with antioxidant potential; thus its intake can contribute for a healthy and well-balanced diet.

Keywords: nutritional profile, amino acids, functional foods, fatty acids, sea cucumbers

1. Experimental

1.1. Sample collection and genetic identification

Adult individuals of *H. arguinensis* were collected in the Ria Formosa (Praia de Faro, Portugal) during autumn / winter of 2011 and species identification was carried out according to traditional taxonomic characters and DNA barcoding. Cytochrome *c* oxidase I (COI) gene was amplified using the protocols from González-Wangüemert and Borrero-Pérez (2012) and sequence was recorded in the GenBank database (GU797739). Fresh adult individuals were gutted, all inner organs removed and the body walls rinsed with fresh water. Samples were frozen at -20°C, freeze-dried for 48h, ground into a fine powder, pooled and stored at -20°C in tightly closed plastic bags until analyses. Each sample corresponds to a minimum of ten adult individuals, with lengths of approximately 20-30 cm. A minimum of three replicates was used for the assays.

1.2. Proximate composition

Moisture was determined by drying the samples at 52°C ($\pm 1^\circ\text{C}$) until constant weight. Ash was determined by incineration in a muffle furnace at 525°C until completely burned (AOAC, 1990), and the crude protein content ($\text{N} \times 6.25$) estimated by the macro-Kjeldahl method. Crude fat was determined by the method of Bligh and Dyer slightly modified (Pereira et al., 2013). Carbohydrates were calculated by difference. Results are expressed as g per 100g of wet weight biomass (WW). Whenever needed, results are also expressed as g per 100g of dry weight biomass (DW). Metabolizable energy (ME) was calculated using the Atwater specific factor for fish (FAO, 2002) according to the following equation: $\text{ME (kcal)} = 4.27 \times (\text{g protein}) + 4.11 \times (\text{g carbohydrate}) + 9.02 \times (\text{g lipid})$. ME was expressed as kcal/100 g of WW.

1.3. Amino acids content

Total amino acids were determined by gas chromatography / mass spectrometry (GC/MS) after sample derivatization with N-methyl-N-tert-butyldimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) according to Deng et al. (2005). Samples (1 μL) were injected at 260°C on an Agilent GC-MS, with a temperature profile of 50°C (1 min) and 10°C min^{-1} to 300°C (6 min). In order to identify and quantify the amino acids, the total ion mode was used. A set of standards of four different concentrations containing all 22 proteinogenic amino acids as well as a known concentration of an internal standard was prepared and measured, and calibration

curves generated for each amino acid. Results are expressed as percentage of total amino acid content.

1.4. Fatty acid methyl esters (FAME) analyses

Lipids and free fatty acids (FA) were converted to the corresponding FAME and analysed on an Agilent GC-MS as described previously (Pereira et al., 2013). Values were expressed in terms of percentage of total fatty acids (FA).

1.5. Minerals content

Minerals were analysed by atomic absorption spectrometry (AAS; GBC Avanta Sigma, Australia) provided with a deuterium background correction. Magnesium (Mg), sodium (Na), potassium (K), calcium (Ca), iron (Fe), manganese (Mn) and zinc (Zn) were analysed by flame AAS with an air-acetylene flame. Cadmium (Cd), chromium (Cr), nickel (Ni) and lead (Pb) were analysed with electrothermal atomisation (GBC graphite furnace 3000) using an auto-sampler (PAL 3000). The accuracy of the analytical procedure was assessed by the analysis of certified reference material, using Community Bureau of Reference BCR60 (*Lagarosiphon major*). Procedural blanks always accounted for less than 1% of the metal concentrations in samples. Values were expressed as g/kg dry weight (DW) (Ca, Mg, Na and K) or mg/kg dry biomass (Fe, Mn, Zn, Cr, Pb, Ni and Cd).

1.6. Preparation of the extracts

Dried samples (1 g) were mixed with hexane (10 mL) and disrupted using a disperser IKA T10B Ultra-Turrax at room temperature (RT). Samples were then centrifuged (5000 x g, 10 min, RT) and the supernatants recovered. The extraction was repeated twice and the supernatants combined and filtered (Whatman n° 4). The residue was then sequentially extracted with diethyl ether, dichloromethane, ethyl acetate, 80% ethanol and water. Additionally, cold and hot water extracts were also prepared. For the cold water extraction, samples (1 g) were mixed with 10 mL of water at RT and disrupted with an Ultra-Turrax. The resulting extract was centrifuged and filtered. Hot water extracts were prepared by stirring samples (1 g) with 10 mL of water at 85°C ($\pm$ 5°C) for 90 min. Organic extracts were dried under reduced pressure at 40°C while the water extracts were freeze-dried. Dried extracts were dissolved in dimethyl sulfoxide (DMSO) at the concentration of 50 mg/mL, aliquoted and stored (4°C).

1.7. Determination of total phenolic content and in vitro antioxidant properties

Total phenolic content (TPC) was evaluated in samples at the concentration of 10 mg/mL, while radical scavenging and metal chelation activities used extracts at concentrations ranging from 1 to 25 mg/mL. Absorbances were read on a microplate reader (Biotek Synergy 4). TPC was assessed by the Folin-Ciocalteu (F-C) method (Julkunen-Tiitto, 1985; Velioglu et al., 1998). Absorbance was read at 725 nm and results were expressed in gallic acid equivalents (GAE) in milligrams per 100 grams of initial dry biomass, using a calibration curve of gallic acid standard solutions. The radical scavenging activity (RSA) on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was evaluated by the assay described by Moreno et al. (2006). The absorbance was measured at 515 nm and RSA was calculated as percent inhibition relative to a blank containing DMSO and as half maximal inhibitory concentration (IC₅₀, mg/mL). Butylated hydroxytoluene (BHT) was used as a positive control at the concentration of 1 mg/mL. The iron (ICA) and copper (CCA) chelating activities were measured according to Megías et al. (2009), and change in colour was measured at 562 nm. The synthetic metal chelator ethylenediaminetetraacetic acid (EDTA) was used as a standard at the concentration of 1 mg/mL.

1.8. Statistical analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM) and experiments were conducted at least in triplicate. Results were subjected to one-way analysis of variance and significant differences in the different nutrients / parameters analysed for *H. arguinensis* was determined by Student-Newman-Keuls posthoc-test ($p < 0.05$) (software “R”, Version 2.13.0, R Development Core Team 2011) with the packages GAD (Sandrini-Neto and Camargo, 2011) and CAR (Fox and Weisberg, 2011).

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Tables captions

Table S1. Extraction yields (%), total phenolics content (TPC, mg GAE/100g dry biomass), and radical scavenging (RSA) and metal chelating activities on iron (ICA) and copper (CCA) (IC₅₀, mg/mL) of extracts of the body wall of *Holothuria arguinensis*.

Tables

Table S1.

Extract/compound	Yield	TPC	RSA	ICA	CCA
<i>Sequential extraction</i>					
Hexane	0.87	n.d.	42.7 ± 3.1^f	> 50	> 50
Diethyl Ether	0.33	3.83 ± 0.35^b	21.9 ± 2.2^e	> 50	> 50
Dichloromethane	0.29	n.d.	22.7 ± 0.9^e	> 50	> 50
Ethyl acetate	0.16	0.29 ± 0.11^c	26.3 ± 2.3^e	> 50	> 50
80% Ethanol	8.22	n.d.	10.8 ± 0.3^c	> 50	12.4 ± 0.4^{bc}
Water	2.07	7.51 ± 1.63^a	18.1 ± 3.3^d	> 50	> 50
<i>One step extraction</i>					
Hot Water	8.66	9.29 ± 4.48^a	8.4 ± 0.7^b	17.3 ± 1.7^c	11.4 ± 0.1^b
Cold Water	14.3	14.2 ± 8.24^a	10.3 ± 1.0^c	11.4 ± 0.1^b	10.8 ± 0.1^b
BHT*			0.1 ± 0.0^a		
EDTA*				0.10 ± 0.00^a	0.28 ± 0.01^a

¹Data represent the mean $\pm$ SEM ($n = 3$). In the same column, values followed by different letters are significantly different at $p < 0.05$ (one-way ANOVA with Student-Newman-Keuls post-hoc test);

*positive control; BHT: Butylated hydroxytoluene, synthetic antioxidant compound; used as a food additive (E321); EDTA: Ethylenediaminetetraacetic acid (chelating agent); n.d: not detected.