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Production and bioaccessibility of *Emiliania huxleyi* biomass and bioactivity of its aqueous and ethanolic extracts

B. Vicente[1] · J. Matos[2,3] · R. Gomes[3] · M. Sapatinha[3] · Cláudia Afonso[3,4] · T. Rodrigues[5] · A. Amorim[1,2] · N. M. Bandarra[3,4] · Carlos Cardoso[3,4]

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Abstract

The coccolithophore *Emiliania huxleyi* (Lohmann) W.W. Hay & H.P. Mohler has been receiving ever increasing attention as a result of its bioactivity potential. A strain of *E. huxleyi* recently isolated from Portuguese coastal waters (NE Atlantic) was cultivated under controlled laboratory conditions and the attained biomass was biochemically characterized (fatty acid (FA) profile, total phenolic and alkenone contents) and its bioactivity measured (antioxidant and anti-inflammatory properties). Moreover, bioaccessibility of selected constituents and bioactivities was assessed. A total dry weight of 556 mg L^{-1} was attained. The biomass was characterized by a large polyunsaturated fatty acids (PUFA) share of the total FA, 60.8 ± 1.8%, followed by saturated fatty acids (SFA), 22.8 ± 1.0%, and monounsaturated fatty acids (MUFA), 15.9 ± 0.7%. Furthermore, an ω3/ω6 ratio of 37 was calculated. The daily amount of freeze-dried *E. huxleyi* required to meet the EPA + DHA recommended daily intake (RDI) was 19.9 g. As to the phenolic content, both extracts had similar values, reaching 435.5–465.4 mg GAE (100 g dw)$^{-1}$. Substantial antioxidant activity as measured by DPPH (2,2-diphenyl-1-picrylhydrazyl), FRAP (ferric reducing antioxidant power), and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was observed. The levels of anti-inflammatory activity were also substantial, 38 ± 4% and 85 ± 11% of COX-2 inhibition in the aqueous and ethanolic extracts, respectively. Moreover, it was observed that the C37:3, C37:2, and C38:2 alkenones were the most abundant, with values exceeding 500 mg (100 g dw)$^{-1}$ and total alkenone content was approximately 2,500 mg (100 g dw)$^{-1}$. The bioaccessibility of studied compounds and bioactivities was always low or even nonexistent/undetected. Therefore, future work should aim at microalgal extraction and processing for achieving higher bioaccessibility levels.

Keywords *Emiliania huxleyi* · Haptophyta · FA composition · Alkenones · Antioxidant activity · Anti-inflammatory activity · Bioaccessibility

✉ Cláudia Afonso
cafonso@ipma.pt

✉ Carlos Cardoso
carlos_l_cardoso@hotmail.com

[1] MARE - Centro de Ciências Do Mar E Do Ambiente, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

[2] Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 16, 1749-016 Lisboa, Portugal

[3] Division of Aquaculture, Upgrading, and Bioprospection (DivAV), Portuguese Institute for the Sea and Atmosphere (IPMA, IP), Avenida Alfredo Magalhães Ramalho, 6, 1495-165 Algés, Portugal

[4] CIIMAR, Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal

[5] Division of Marine Geology and Georesources (DivGM), Portuguese Institute for the Sea and Atmosphere (IPMA, IP), Avenida Alfredo Magalhães Ramalho, 6, 1495-165 Algés, Portugal

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Introduction

The coccolithophore *Emiliania huxleyi* (Lohmann) W.W. Hay & H.P. Mohler (Haptophyta, Coccolithophyceae, Isochrysidales, Noelaerhabdaceae) is deemed a potentially relevant source of biomolecules (Aveiro et al. 2020). In particular, the neutral lipids of *E. huxleyi* have attracted considerable attention over the last 25 years, but also its polar lipids have been studied (Conte et al. 1994; Bell and Pond 1996; Eltgroth et al. 2005). Within ω3 polyunsaturated fatty acids (PUFA) high relative levels of stearidonic acid (18:4 ω3), octadecapentaenoic acid (18:5 ω3), and docosahexaenoic acid (DHA, 22:6 ω3) have been reported (Conte et al. 1994; Bell and Pond 1996). DHA is deemed beneficial for the neural development of children (Cardoso et al. 2018) and for the prevention of mild cognitive decline in elderly (Cardoso et al. 2016). As other microalgae, *E. huxleyi* is known to accumulate phenolic compounds with antioxidant activity (Jin et al. 2015). It is also worth mentioning that the biomass of another haptophyte, *Isochrysis galbana* Parke, may have anti-inflammatory properties (Nuño et al. 2013). If the biomass of *E. huxleyi* displays this and other bioactivities as well as key nutrients, it may be considered as a potential nutraceutical.

Any future application of *E. huxleyi* biomass as nutraceutical would have to consider the absorbability of any given biomolecule in the gastrointestinal (GI) tract. For this purpose, bioaccessibility would need to be assessed. Bioaccessibility corresponds to the part of the initial biomolecule content that is freed from the microalgal structure into the GI tract (Afonso et al. 2015). Hence, the evaluation of the nutraceutical potential of *E. huxleyi* should consider the assessment of bioaccessibility. Such assessment entails the utilization of an appropriate in vitro digestion model for a realistic simulation of human digestion. Various procedures have been developed (Cardoso et al. 2015) and optimized (Afonso et al. 2015; Cardoso et al. 2015), including the static model with digestive compartment distinction and complete digestive juices.

This study targeted the determination of the FA, total phenolic, and alkenone composition as well as the antioxidant and anti-inflammatory activities in aqueous and ethanolic extracts of *E. huxleyi*. In addition, the bioaccessibility of the studied compounds was evaluated, thereby enabling an assessment of the nutraceutical potential of this highly relevant coccolithophore.

Materials and methods

Microalgal production

The strain of *Emiliania huxleyi* (IO30-03) used in the present study was isolated in October 2018 from water samples collected in the coast of Sesimbra, Portugal (38°26′9.26″N 9°3′38.99″W) by single cell isolation under an inverted light microscope as described in Andersen and Kawachi (2005). Single cells were inoculated in 24-well culture microplates (Thermo Scientific Nunc 176,740) filled with filter-sterilized seawater and incubated in a culture chamber at 15 ± 1 °C (Fitoclima S600, Aralab, Portugal) under 50 µmol photons m^{-2} s^{-1} provided by cool white fluorescent light bulbs (18 W, Osram, Germany) on a 12:12 light/dark cycle. The resulting culture is monospecific, but non-axenic.

The culture was scaled-up in f/2 culture medium (Guillard 1975) in 16 batches of 1250 mL, using low-density polyethylene (LDPE) sleeves under the following experimental settings: 22 ± 1 °C; 240 µmol photons m^{-2} s^{-1} cool white fluorescent light (18 W, Osram, Germany) with a 12:12 light/dark cycle. Cells were kept in suspension by aeration with sterilized (0.2 µm) air. The total volume produced was 20.0 L.

Culture monitoring

Cultures were monitored by in vivo fluorescence (Water-PAM, Walz, Germany) and by cell counts using a Burker counting chamber.

Subsamples of each replicate (3.5 mL) were collected on a daily basis, at the same time of the day, and were dark-adapted at room temperature for 10 min. The ratio variable fluorescence (F_v)/maximum fluorescence (F_m), a measure of maximal PSII photochemical efficiency, was used as an indicator of the physiological state of cells in the culture (Kromkamp et al. 2008).

For cell counting, subsamples that were used to assess in vivo fluorescence were fixed with neutral Lugol's solution and counted every 2 days at 125 × magnification (Zeiss Axioskop, Germany). A minimum of 400 cells was counted to ensure statistical significance. Growth rates (day^{-1}) were calculated according to Wood et al. (2005).

Scanning electron microscopy (SEM)

For SEM, an aliquot of the culture was filtered by gravity onto a 3-µm polycarbonate membrane (Whatman WHA110612). The filter was then washed with distilled water, neutralized with NaOH (1 M) to remove salt, and left to dry at room temperature. Filters were sputter coated with gold and examined using a JEOL JSM 5200LV microscope (JEOL, Japan).

Sample preparation

Microalgal biomass was collected at mid-late exponential growth phase by centrifugation at 2,615 × *g* for 25 min. The supernatant was then discarded and the cell pellets

were pooled and recovered in 50-mL falcon tubes (Thermo Scientific Nunc, 339,652). A second centrifugation step of 2,150 × *g*, 25 min, was applied. The resulting pellets were freeze-dried and stored in cold (− 80 °C) until further analysis.

Fatty acid profile

Fatty acid methyl esters (FAMEs) were prepared by acid-catalysed transesterification using the methodology described by Bandarra et al. (1997). Samples were injected into a Varian Star 3800 CP gas chromatograph (USA), equipped with an auto sampler with a flame ionization detector at 250 °C. FAMEs were identified by comparing their retention time with those of Sigma-Aldrich standards (PUFA-3, Menhaden oil, and PUFA-1, marine source from Supelco Analytical). The LOD is 1 mg $(100\ g)^{-1}$. The quantification of total FA was based on the internal standard technique, using the heneicosanoic acid (21:0) methyl ester (Sigma-Aldrich). All analytical determinations were made in triplicate.

Phenolic content and antioxidant activity

Total phenolic content

For extracting phenolic substances, water and ethanol were chosen as solvents (Siriwoharn et al. 2004). The microalgal sample (or the evaporated bioaccessible extract) was weighed (1.25 g), homogenized with 25 mL of solvent using a model Polytron PT 6100 homogenizer (Kinematica, Switzerland) (30,000 rpm, 1 min), and put for 18 h on an orbital shaker (400 rpm). The attained mixture was centrifuged (3,000 × g, 4 °C, 10 min) and the supernatant was filtered to a final volume of 25 mL. Afterwards, the Singleton and Rossi (1965) technique was applied to this extraction solution. Gallic acid (GA) was the standard and polyphenol concentration was determined as gallic acid equivalent (mg GAE $(100\ g\ dw)^{-1}$) with this standard's calibration curve.

Antioxidant activity as measured by the DPPH method

The DPPH (2,2-diphenyl-1-picrylhydrazyl) method was performed as described by Miliauskas et al. (2004). Extracts were prepared as in the "Total phenolic content" section. Based on an ascorbic acid calibration curve, results were expressed in mg of ascorbic acid equivalents (mg AA Eq. $(100\ g\ dw)^{-1}$).

Antioxidant activity as measured by the FRAP method

A modified version of Benzie and Strain (1996) was applied for the determination of FRAP (ferric reducing antioxidant power). Extracts were prepared as in the "Total phenolic content" section. Based on a $FeSO_4$ standard curve, results were calculated as μmol Fe^{2+} $(g\ dw)^{-1}$.

Antioxidant activity as measured by the ABTS method

ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was quantified by the Re et al. (1999) procedure. Extracts were prepared as in the "Total phenolic content" section. The ABTS radical scavenging activity of the samples was calculated as μmol of Trolox equivalents (μmol Trolox Eq) $(100\ g\ dw)^{-1}$ using a Trolox calibration curve.

Alkenone content

The analytical procedure to determine total alkenone concentration of 37, 38, and 39 carbon atoms is described elsewhere (Villanueva et al. 1997; Rodrigues et al. 2009; Salvadó et al. 2012). A mix of internal standard of n-nonadecan-1-ol, n-hexatriacontane, and tetracontane was added to a 19.3 mg of dry microalgal sample in a centrifuge tube of 10 mL. The whole extract was hydrolysed with 3 mL of 6% potassium hydroxide and purified using n-hexane. The purified extracts were derivatized with BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) diluted with 50% toluene (12 h at room temperature). Sample was quantified with Varian 3800 gas chromatograph equipped with a septum programmable injector, flame ionization detector, and CPSIL-5 CB column (coated with 100% dimethylsiloxane; film thickness of 0.12 μm; L (m) × ID (mm) × OD (mm): 50 × 0.32 × 0.45). Hydrogen was the carrier gas (2.5 mL min^{-1}). Alkenone concentrations were quantified using n-hexatriacontane as internal standard.

The $U^{k'}_{37}$ index was calculated as follows:

$$U^{k'}_{37} = [C37:2]/([C37:2] + [C37:3])$$

where:

[C37:2] content of the alkenone C37:2 in the microalgal biomass;

[C37:3] content of the alkenone C37:3 in the microalgal biomass.

Moreover, the corresponding temperature (T) during the growth of the microalgal biomass was estimated in accordance to the formula:

$$T = (U^{k'}_{37} - 0.044)/0.033$$

where *T*—temperature of microalgal growth expressed in °C.

Anti-inflammatory activity

Aqueous extract preparation for in vitro *anti-inflammatory activity*

Aqueous and ethanolic extracts were prepared from the freeze-dried samples of *E. huxleyi*, with the purpose of attaining fractions with anti-inflammatory properties to be tested in vitro.

Accordingly, approximately 200 mg of freeze-dried *E. huxleyi* was weighed and homogenized with 2 mL of Milli-Q water or absolute ethanol using a model Polytron PT 6100 homogenizer (Kinematica, Switzerland) at a velocity of 30,000 rpm for 1 min. The mixture was subjected to a thermal treatment (at 80 °C for 1 h). Both the microalgae and bioaccessible extraction mixtures were centrifuged ($3{,}000 \times g$ at 4 °C for 10 min) and the respective supernatant was evaporated using vacuum rotary evaporator with the water bath temperature at 65 °C and inert gas (nitrogen) stream.

Cyclooxygenase (COX-2) inhibition assay

The prepared extracts were dissolved in 100% DMSO to prepare a stock preparation with a concentration of 10 mg mL^{-1}. The extract was tested at 1 mg mL^{-1} using a commercial cyclooxygenase (COX) inhibitory screening assay kit, Cayman test kit-560131 (Cayman Chemical Company, USA). The COX inhibitor screening assay directly measures the amount of prostaglandin F2α generated from arachidonic acid (AA, 20:4 ω6) in the cyclooxygenase reaction. A volume of 10 µL of test extract or DMSO was used. The reaction was initiated by addition of 10 µL 10 mM AA and each reaction tube was incubated at 37 °C for 2 min. Reaction was terminated by addition of 50 µL 1 N HCl and saturated stannous chloride. Assays were performed using 100 units of human recombinant COX-2. An aliquot was removed and the prostanoid produced was quantified spectrophotometrically (412 nm) via enzyme immunoassay (ELISA) after 18 h incubation, washing, addition of Ellman's reagent, and further 90 min incubation. Interference by solutions and digestive enzymes used in the bioaccessibility method was taken into account by subtracting COX-2 inhibition of the bioaccessibility blank from the COX-2 inhibition measured with the bioaccessible fraction samples.

In vitro digestion model

An in vitro digestion model was chosen for the determination of bioaccessibility in freeze-dried *E. huxleyi*. This model comprises three sections, which enable the simulation of digestion in three different parts of the GI tract: mouth, stomach, and small intestine. The solutions and enzymes used in this model followed Afonso et al. (2015). Briefly, approximately 1.5 g freeze-dried *E. huxleyi* was weighed taking into account the assumptions defined by Versantvoort et al. (2005). Sample was mixed with 4 mL of artificial saliva at a pH 6.8 ± 0.2 for 5 min, then 8 mL of artificial gastric juice (pH 1.3 ± 0.02 at 37 ± 2 °C) was added, and pH was lowered to 2.0 ± 0.1. The mixing lasted 2 h in a head-over-heels movement (37 rpm at 37 ± 2 °C). Finally, 8 mL of artificial duodenal juice (pH 8.1 ± 0.2 at 37 ± 2 °C), 4 mL of bile (pH 8.2 ± 0.2 at 37 ± 2 °C), and 1.33 mL of HCO_3^- solution (1 M) were added. The pH of the mixture was set at 6.5 ± 0.5 and agitation for 2 h was identical to gastric conditions. The mixture generated in the in vitro model was subjected to centrifugation at $2750 \times g$ for 5 min, thus yielding a non-digested portion and the bioaccessible fraction. Chemicals were supplied by Merck (Germany); enzymes were obtained from Sigma (USA).

Calculation of bioaccessibility

The percentage (%) of each *E. huxleyi* constituent (C)—or bioactivity—in the bioaccessible fraction was estimated as follows:

$$\%\ \mathrm{C\ bioaccessible} = [\mathrm{C}]_{\mathrm{bioaccessible}} \times 100/[\mathrm{S}]$$

where [C] = concentration of constituent, and [S] = [C] in the initial sample (prior to digestion).

Statistical analysis

To test the normality and the homogeneity of variance of data, the Kolmogorov–Smirnov's test and Levene's F-test, respectively, were used. Data, which corroborate these assumptions, were analysed by one-way ANOVA distribution using the Tukey HSD to determine the difference in the constituent content or bioactivity between extracts and with respect to the bioaccessible fraction. For all statistical tests, the significance level (α) was 0.05. All data analysis was performed using STATISTICA 6 (Stat-soft, Inc. USA 2003).

Results

Production of *Emiliania huxleyi* biomass

The culturing sleeves used for biomass production of *E. huxleyi* were inoculated from a culture in exponential growth phase (day 8) (Fig. 1A). Following inoculation, the cultures quickly attained exponential growth phase (Fig. 1B) with an average specific growth rate of 0.6 day^{-1}. The biomass was collected on day 8 when the maximum cell concentration was attained

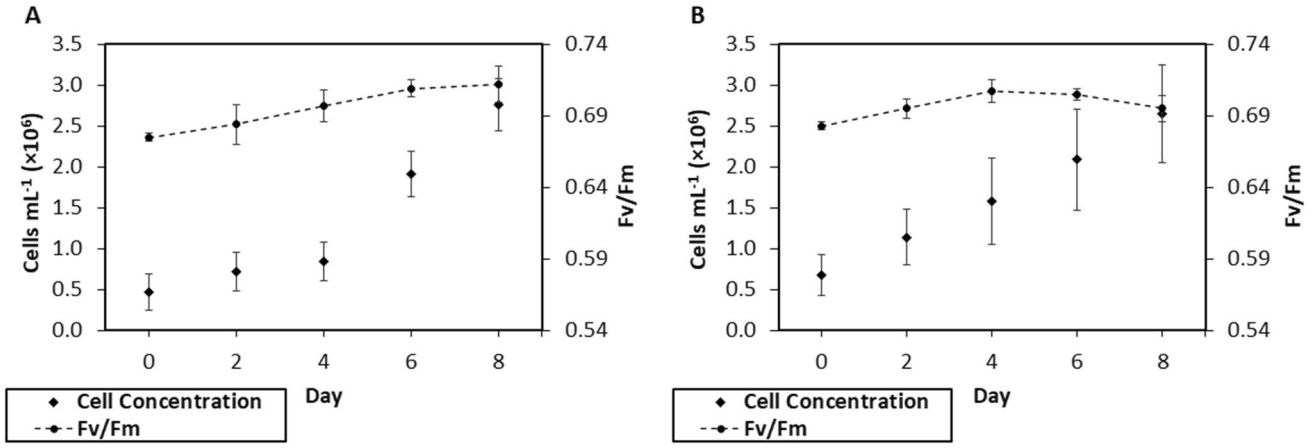

Fig. 1 Growth curves of the cultivated strain of *Emiliania huxleyi* (IO30-03). **A** Inoculum production culture. **B** Biomass production culture. F_v/F_m, maximum quantum yield in PSII

$(2.69 \pm 0.61 \times 10^6$ cells $mL^{-1})$ (Fig. 1B). The high F_v/F_m value (Fig. 1B) indicated good physiological status at the time of harvest. The achieved total productivity was 556 mg dw L^{-1} (total biomass 11.2 g dw). A SEM image of the cultivated strain is shown in Fig. 2.

Fatty acid composition

The fatty acid composition of *E. huxleyi* is presented in Table 1.

Concerning the relative FA profile, there are two key issues that must be highlighted: first, PUFA share in the total FA was high, $60.8 \pm 1.8\%$, thus outstripping the SFA share, $22.8 \pm 1.0\%$, and the monounsaturated FA (MUFA) share, $15.9 \pm 0.7\%$; second, within the PUFA group, ω3 PUFA were much more abundant than ω6 PUFA, thus leading to a ω3/ω6 ratio of 37. At a more detailed level, while myristic acid (14:0) was the main SFA ($14.1 \pm 0.9\%$ of the total FA), oleic acid (18:1 ω9) was the dominant MUFA ($11.1 \pm 0.5\%$). Regarding PUFA, three main FAs were very abundant and all of which were ω3 PUFA, stearidonic acid, octadecapentaenoic acid, and DHA, with each exceeding 14% of the total FA. In contrast, the eicosapentaenoic acid (EPA, 20:5 ω3) level was low, $2.0 \pm 0.4\%$, and the alpha-linolenic acid (18:3 ω3) was undetected. In absolute terms (mg (100 g dw)$^{-1}$), total FA content reached 12,140 mg (100 g dw)$^{-1}$ and there were five FAs exceeding 1,000 mg (100 g dw)$^{-1}$, namely, myristic, oleic, stearidonic, and octadecapentaenoic acids as well as DHA. In particular, DHA content was quite substantial, $2{,}269 \pm 85$ mg (100 g dw)$^{-1}$.

Phenolic content and antioxidant activity

The phenolic content and antioxidant activity as measured by DPPH, FRAP, and ABTS methodologies in aqueous and ethanolic extracts of *E. huxleyi* are shown in Table 2.

Regarding the phenolic content, there were no statistical differences between the two types of extracts, reaching values in the range 435.5–465.4 mg GAE (100 g dw)$^{-1}$. This situation was nearly replicated by the FRAP values, but with a significantly higher antioxidant activity in the ethanolic extract, 47.8 ± 1.4 µmol Fe^{2+} Eq (g dw)$^{-1}$, 33% higher than the aqueous extract's value. This difference between the antioxidant activities of the extracts was larger when measured by ABTS, reaching 82%, but with higher

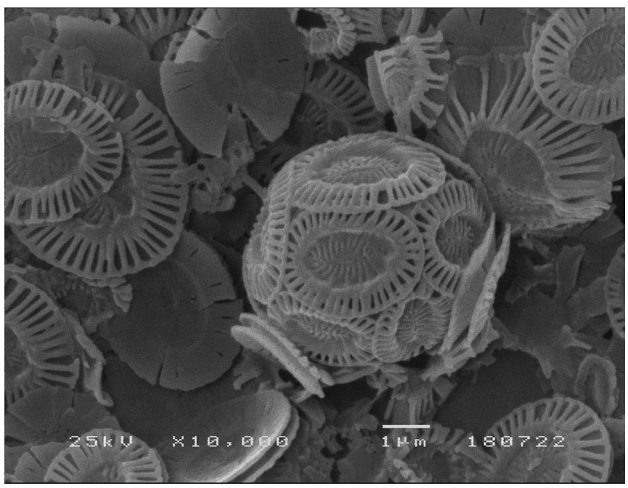

Fig. 2 Scanning electron microscopy (SEM) image of the cultivated strain of *Emiliania huxleyi* (IO30-03)

Table 1 Fatty acid profile (in % of total fatty acids and in mg (100 g dry weight)$^{-1}$) of the studied microalgae *E. huxleyi*

Fatty acid	% total fatty acids	mg (100 g dry weight)$^{-1}$
14:0	14.1 ± 0.9	1,720 ± 110
16:0	7.1 ± 0.3	866 ± 37
18:0	1.3 ± 0.2	159 ± 24
Σ SFA	22.8 ± 1.0	2,782 ± 122
16:1 ω7	4.4 ± 0.2	537 ± 24
18:1 ω9	11.1 ± 0.5	1,354 ± 61
Σ MUFA	15.9 ± 0.7	1,940 ± 85
18:2 ω6	1.6 ± 0.1	195 ± 12
18:3 ω3	ND	ND
18:4 ω3	14.6 ± 0.6	1,781 ± 73
18:5 ω3	23.7 ± 0.5	2,891 ± 61
20:5 ω3	2.0 ± 0.4	244 ± 49
22:6 ω3	18.6 ± 0.7	2,269 ± 85
Σ PUFA	60.8 ± 1.8	7,418 ± 220
Σ ω3	59.2 ± 1.7	7,222 ± 207
Σ ω6	1.6 ± 0.1	195 ± 12
Σ ω3/Σ ω6	37.0 ± 1.5	37.0 ± 1.5

Values are presented as average ± standard deviation. *ND* not detected

activity in the aqueous extract, 6,221 ± 180 µmol Trolox Eq. (100 g dw)$^{-1}$. Finally, DPPH exhibited the sharpest contrast between extracts, since no activity was measured in the ethanolic extract vs 47.2 ± 0.2 mg AA Eq. (100 g dw)$^{-1}$ in the aqueous extract.

Alkenones

The alkenone profile of the studied microalgae *E. huxleyi* can be found in Table 3. The microalgal biomass was rich in some alkenones, namely, C37:3, C37:2, and C38:2, all exceeding 500 mg (100 g dw)$^{-1}$. The C39 alkenones had a much lower contribution, not surpassing 24 mg (100 g dw)$^{-1}$. The UK_{37} index reached a value of 0.62 and the total alkenone content exceeded 2,500 mg (100 g dw)$^{-1}$.

Anti-inflammatory activity

The anti-inflammatory activity values of the aqueous and ethanolic extracts of *E. huxleyi* expressed as a percentage of inhibition of the enzyme COX-2 are shown in Table 4. Extract concentration was 1 mg mL^{-1} in DMSO. The levels of anti-inflammatory activity were different, ranging from 38 ± 4% of COX-2 inhibition in the aqueous extract to 85 ± 11% of COX-2 inhibition in the ethanolic extract.

Table 2 Phenolic content (in mg GAE (100 g dry weight)$^{-1}$) and antioxidant activity as measured by DPPH (mg AA Eq. (100 g dw)$^{-1}$), FRAP (µmol Fe^{2+} Eq (g dw)$^{-1}$), and ABTS (µmol Trolox Eq. (100 g dw)$^{-1}$) methods in aqueous (Aq.) and ethanolic (Eth.) extracts and in the bioaccessible fraction of the studied microalga *E. huxleyi*

Parameter	Extract	*E. huxleyi*
Total phenolic content (mg GAE (100 g dw)$^{-1}$)	Aqueous	435.5 ± 19.4^{a}
	Ethanolic	465.4 ± 30.0^{a}
	Bioacc	ND^{b}
DPPH (mg AA Eq. (100 g dw)$^{-1}$)	Aqueous	47.2 ± 0.2^{a}
	Ethanolic	ND^{c}
	Bioacc	2.3 ± 0.5^{b}
FRAP (µmol Fe^{2+} Eq (g dw)$^{-1}$)	Aqueous	35.9 ± 0.3^{b}
	Ethanolic	47.8 ± 1.4^{a}
	Bioacc	ND^{c}
ABTS (µmol Trolox Eq. (100 g dw)$^{-1}$)	Aqueous	6,221 ± 180^{a}
	Ethanolic	3,417 ± 154^{b}
	Bioacc	ND^{c}

Values are presented as the average ± standard deviation. *ND* not detected. Different lowercase letters within a column correspond to significant differences ($p < 0.05$) between extracts (aqueous, ethanolic, bioaccessible fraction) for each parameter

Bioaccessibility

The bioaccessible content of the various constituents of the microalgal biomass and the bioaccessible bioactivities were very low or undetected, as can be seen in Tables 2, 3, and 4. It was not possible to determine the FA composition in the bioaccessible fraction, since almost no fat was rendered bioaccessible. Only DPPH revealed a significant antioxidant bioactivity in the bioaccessible fraction, 2.3 ± 0.5 mg AA Eq. (100 g dw)$^{-1}$. This value was compared to the aqueous extract of the initial (prior to digestion) biomass since the digestion is a very specific aqueous extraction. Such comparison showed that only a fraction of the original antioxidant power became bioaccessible. Regarding other measured

Table 3 Alkenone profile (mg (100 g dry weight)$^{-1}$) of the studied microalgae *E. huxleyi*

Alkenone	mg (100 g dry weight)$^{-1}$
C37:3	591.4
C37:2	959.8
C38:3	237.8
C38:2	691.7
C39:3	3.7
C39:2	23.6
$U^{K'}_{37}$	0.62
Total alkenones	2,508.1

Values are presented as average

Table 4 Anti-inflammatory activity (% inhibition of COX-2) in aqueous (Aq.) and ethanolic (Eth.) extracts and in the bioaccessible fraction of the studied microalgae *E. huxleyi*

	Extract	Anti-inflammatory activity (% inhibition of COX-2)
Initial	Aqueous	38 ± 4^{b}
	Ethanolic	85 ± 11^{a}
Bioaccessible		ND[c]

Values are presented as mean ± standard deviation. *ND* not detected. Different lowercase letters within a column correspond to statistical differences ($p < 0.05$)

properties, for the bioaccessible fraction extract—after eliminating the bioaccessibility blank background interference—, no activity was detected.

Discussion

The current study aimed to provide an overall assessment of key bioactive compounds and bioactivities in *E. huxleyi*. The focus was on the lipophilic components, namely, fatty acids, phenolic compounds, and alkenones, which constitute a large part of the lipophilic fraction of the biomass, and their associated bioactivities.

Fatty acid composition

The FA composition of *E. huxleyi* showed a significant diversity of FAs and indicated that this group of lipid compounds represents a large portion of the lipophilic fraction of the microalgal biomass. Indeed, a total FA content of 12,140 mg (100 g dw)$^{-1}$ compares to 16,945 mg of total lipid content per 100 g dw. These results are in agreement with previous work where *E. huxleyi* has been reported to contain high relative levels of stearidonic acid, octadecapentaenoic acid, and DHA (Conte et al. 1994; Bell and Pond 1996). These three ω3 PUFA are also among the main PUFA in other studies on *E. huxleyi* (Jónasdóttir 2019). In particular, the high levels of the uncommon octadecapentaenoic acid in this species are noteworthy. This FA is very concentrated in the digalactosyldiacylglycerol lipid fraction (Bell and Pond 1996). However, other authors (Fiorini et al. 2010) have found low levels of this FA in the phospholipid fraction. DHA is almost always a major ω3 PUFA in the various lipid fractions of *E. huxleyi* (Bell and Pond 1996; Fiorini et al. 2010; Jónasdóttir 2019). Furthermore, according to literature (Bell and Pond 1996; Fiorini et al. 2010; Jónasdóttir 2019), within MUFA and SFA, oleic acid and myristic acid are the most meaningful, respectively. In contrast with these results, a recent study (Aveiro et al. 2020) reported that stearic acid (16:0) was the main SFA in *E. huxleyi*. However, this finding was related to the polar lipids. In addition, while DHA was also abundant (17.2 ± 2.7%), octadecapentaenoic acid had a lower content (6.6 ± 0.5%), thus reinforcing the apparent fact that it is less abundant among polar lipids (Aveiro et al. 2020).

Although *I. galbana* is not taxonomically distant from *E. huxleyi*, there are substantial differences in the FA profile, which in *I. galbana* may be poorer in octadecapentaenoic acid and DHA (Bonfanti et al. 2018). One aspect that should be considered when investigating the FA profile of *E. huxleyi* is that it can vary widely as a function of cultivation conditions and growth phase at the harvesting time (Fiorini et al. 2010). For instance, higher CO_2 partial pressure during cultivation of *E. huxleyi* has been shown to lead to lower relative contents of DHA, decreasing in the diploid stage from 22.0 to 1.2% of the total phospholipid FA and in the haploid stage from 33.7 to 1.7%.

In the present work, if the absolute concentrations of EPA and DHA are combined, an EPA + DHA content of 2,513 mg (100 g dw)$^{-1}$ is achieved, which is meaningful regarding its health impact (Swanson et al. 2012; Dyall 2015). Indeed, according to the American Heart Association (Kris-Etherton et al. 2002), the recommended daily intake (RDI) of EPA and DHA to achieve human EPA + DHA requirements should be 500 mg day^{-1}. Based on this value, the amount of freeze-dried *E. huxleyi* that would be required to meet the EPA + DHA RDI would correspond to a daily amount of 19.9 g. This value suggests that *E. huxleyi* may be potentially interesting as a nutraceutical added to functional foods, provided that there are no deleterious sensory effects. However, since high EPA and DHA levels prior to digestion do not necessarily translate into an elevated intestinal absorption of these FAs, the lipid digestion must be studied (see "Bioaccessibility" section).

Phenolic content and antioxidant activity

Literature regarding the antioxidant activity of *E. huxleyi*'s extracts is scarce. A substantial level of ABTS activity under aqueous extraction conditions has been determined and related to the specific physiological status of the microalgae (Schieler et al. 2019).

The comparison with the better studied *I. galbana* shows that phenolic contents are similar to those found in the extracts of *E. huxleyi*, 430–515 mg GAE (100 g dw)$^{-1}$ (Matos et al. 2019). Nevertheless, it must be emphasized that antioxidant compound levels may be affected by cultivation and harvesting conditions, such as nutrient availability, temperature, and growth phase (Maadane et al. 2015). There is also some similarity with the ABTS values of *I. galbana* (Matos et al. 2019). However, aqueous and ethanolic extracts

of *I. galbana* did not show large differences between them. When comparison is extended to microalgal species from contrasting groups, there are also similar ABTS values, namely, in *Phaeodactylum tricornutum* (diatom) and *Tetraselmis* sp. (chlorophyte) (Goiris et al. 2012).

Given the antioxidant properties of phenolic compounds, some degree of correlation between DPPH/FRAP/ABTS values and total phenolic content may exist. However, this was not verified in the current study. Only between FRAP levels and phenolic contents some agreement was detectable. A plausible explanation may lie in the biosynthesis of a wide array of distinct antioxidant compounds by microalgae, such as carotenoids (Garrido et al. 2016). Hence, specific compounds or classes of compounds other than phenolic substances may have affected the various measured antioxidant activities. In any case, the isolation and characterization of phenolic components from *E. huxleyi* would be relevant to better understand the link between phenolic substances and antioxidant properties.

The various antioxidant compounds in the biomass *E. huxleyi* may also represent an additional advantage for its use as nutraceutical, provided that the bioaccessibility of the antioxidant compounds is high.

Alkenones

The alkenone profile of studied *E. huxleyi* revealed a higher abundance of C37s than C38s and yielded a $U^{K'}_{37}$ index associated to a temperature of 17.4 °C. As described in the "Microalgal production" section, cultures were grown at 22 ± 1 °C. The divergence between these two temperatures needs further research, but it is out of the scope of the present work. Total alkenone content, 2,508 mg (100 g dw)$^{-1}$, must be compared to total lipid content, 16,945 mg (100 g dw)$^{-1}$, and total FA content, 12,140 mg (100 g dw)$^{-1}$. These values resulting from independent analyses fit together and show that approximately 15% of the total lipid fraction is composed by alkenones. The total FA represents 72%, but, if they belong to the phospholipid sub-fraction, their weight in the total lipid fraction may be higher than 80%. The remaining few percentage points may correspond to non-saponifiable lipids other than alkenones.

A comparison in the literature shows that lipid composition and total weight of the alkenone fraction may vary substantially between *E. huxleyi* isolates (Pond and Harris 1996). For instance, alkenone share of the total lipid fraction may range from 17.9 to 47.5% (Pond and Harris 1996). Bakku et al. (2018) showed that the relative importance of the alkenones in *E. huxleyi* biomass may decrease when nitrogen is not limiting in the culture medium. However, these authors observed a persistent predominance of C37:2 and C38:2 alkenones just as in the current study. Only C37:3 alkenones were much less abundant in the overall alkenone profile of that particular strain of *E. huxleyi* (CCMP 2090 cultivated at 20 °C), not exceeding 10% (Bakku et al. 2018), which contrasts with almost 25% in the present study. Another study, on the haptophyte *Tisochrysis lutea*, which also produces alkenones (Shi et al. 2015), showed the presence of long-chain alkenones (mainly C37 and C38) as major component, representing more than 74% of the total lipid content. Long-chain alkenes, 1.2%, and other neutral lipids were approximately 25%, being FA negligible (Shi et al. 2015). In this case, the lipid fraction differed significantly from that found in the herein studied haptophyte, *E. huxleyi*.

Alkenones may find application in lipsticks, lip balms, and creams, thereby making them a potential wax-like compound for personal care products, especially given their wax compatibility and high melting temperature (71.1–77.4 °C) (McIntosh et al. 2019). Alkenones have been investigated in sunscreens, particularly in combination with three "reef safe" UV filters (O'Neil et al. 2019). Despite all envisaged potential, these and other possible applications still need more research (Moheimani et al. 2012). Alkenones seem to point to cosmetic or cosmeceutical and not to nutraceutical applications. Hence, their bioaccessibility was not studied.

Anti-inflammatory activity

There is already research into the anti-inflammatory activity of microalgae, but there are several alternative approaches being used, encompassing in vitro assays and in vivo models (Renju et al. 2013; Jensen et al. 2015). This renders any literature comparison quite difficult. Nonetheless, various studies have reported anti-inflammatory activity in microalgae (Jensen et al. 2015; Reyes et al. 2016; Bonfanti et al. 2018). While some studies found significant activity in aqueous extracts, for instance, in *Arthrospira platensis* (Jensen et al. 2015), other researchers have claimed anti-inflammatory properties of hydrophobic components, namely, in lipid extracts of *I. galbana* (Reyes et al. 2016; Bonfanti et al. 2018). In particular, Bonfanti et al. (2018) used the same methodology and reported an anti-inflammatory activity of 79 ± 7% of COX-2 inhibition in the lipid extract. This is a high value comparable to that measured in the ethanolic extract of *E. huxleyi*, where hydrophobic substances were also present.

Beside their hydrophobicity, the nature of these substances is unclear and warrants further investigation. In fact, different molecules may be implicated, namely, lipids, phenolic compounds, carotenoids, phytosterols, or alkaloids. Lipids and lipophilic substances are likely candidates as anti-inflammatory bioactives of *E. huxleyi*, given their high concentration (Table 1) and hydrophobicity. Besides EPA, which is reported to display an anti-inflammatory action (Tanaka et al. 2014), other lipophilic components in the

coccolithophore biomass may enhance the anti-inflammatory activity. However, the anti-inflammatory effects of EPA and other ω3 PUFA (Calder 2010) require an in vivo system, an approach that fundamentally differs from the COX-2 inhibition procedure used here with *E. huxleyi*. A recent study (Reyes et al. 2016) recognized glycolipids as exerting an inhibitory effect on the formation of pro-inflammatory cytokines in human macrophages. The COX-2 inhibition in the ethanolic extract—and, to a lesser extent, in the aqueous extract—of *E. huxleyi* suggests a different anti-inflammatory mechanism.

The high anti-inflammatory activity of the studied microalgal biomass also reinforces the possibility of its use as a nutraceutical in functional foods. For this, an in vitro digestive model must be applied to the biomass.

Bioaccessibility

Regarding *E. huxleyi*, there is a significant opposition between the biochemical profile and bioactivity of the microalgal biomass prior to digestion and the bioaccessible fraction after digestion. Such contrast highlights the importance of studying human digestion of microalgal biomolecules and bioactivities. There are few studies on this subject, namely, on the FA in vitro bioaccessibility in the whole microalgal biomass. The percentages of FA bioaccessibility in *E. huxleyi* were very low (undetected bioaccessible FA profile), but have a precedent in *I. galbana*, which yielded FA bioaccessibility levels below 15% (Bonfanti et al. 2018). Moreover, there has been research regarding FA apparent digestibility—calculated on the basis of the FA levels in the diet and faeces, thus providing an estimate of the portion of the initial FA concentration that is absorbed by the organism—using salmon as model (Kousoulaki et al. 2016). These authors used *Schizochytrium* sp., a non-photosynthetic unicellular heterokont and, in this case, they observed high digestibility for ω3 PUFA. This contrasts with the low FA bioaccessibility in the two haptophytes. However, given the high phylogenetic distance between the two groups (heterokonts and haptophytes), such differences may be justified (Gangl et al. 2015).

In addition, the inhibitor(s) of COX-2 and the antioxidants, as measured by FRAP and ABTS, did not seem to be bioaccessible. If a low bioaccessibility of the anti-inflammatory and antioxidant substances in *E. huxleyi* is confirmed, preparation of extracts or microalgal processing through decoction and/or other treatments (Cavonius et al. 2016) may be necessary for increasing bioaccessibility.

There may exist obstacles for the digestion of microalgal biomass. Namely, a study on *Nannochloropsis oculata* (Cavonius et al. 2016) showed protein and lipid accessibility difficulties by the used mammalian digestive enzymes. The cell wall composition of *Nannochloropsis*, composed mainly by cellulose, offered an explanation, given its ability to block enzymes (Scholz et al. 2014). In the case of the coccolithophore *E. huxleyi*, the cell is covered with calcite discs, coccoliths, which dissolve under acidic conditions, such as those experienced during the simulated gastric digestion (pH 2.0 ± 0.1 for 2 h). Hence, future research work concerning Ca in the bioaccessible fraction is warranted.

The reason for the low lipid bioaccessibility in the two haptophytes (*E. huxleyi* and *I. galbana*) may lie elsewhere. In particular, chemical affinity effects could play a role in the bioaccessibility results. For instance, microalgal lipids could interact more strongly with the non-bioaccessible fraction components. There may also be microalgal components that inhibit digestive enzymes, thereby reducing bioaccessibility.

The low bioaccessibility represents a problem if nutraceutical applications for the biomass are envisaged. The antioxidant, anti-inflammatory, and lipid bioactive potential of *E. huxleyi* can only be brought to fruition if bioaccessibility is enhanced. This may involve the pre-treatment—for instance, thermal or enzymatic—of the biomass prior to its incorporation in functional foods or the utilization of extracts rich in lipids, antioxidant, and anti-inflammatory compounds.

Conclusions

The biomass of the studied haptophyte, *E. huxleyi*, was characterized. Results indicated a large PUFA share of the total FA, $60.8 \pm 1.8\%$, followed by SFA, $22.8 \pm 1.0\%$, and MUFA, $15.9 \pm 0.7\%$. Furthermore, an ω3/ω6 ratio of 37 was calculated. The daily amount of freeze-dried *E. huxleyi* that is necessary to meet the EPA + DHA RDI was found to be 19.9 g. As to the phenolic content, both extracts had similar values, reaching 435.5–465.4 mg GAE $(100\ g\ dw)^{-1}$. Moreover, substantial antioxidant activity as measured by DPPH, FRAP, and ABTS was observed. The levels of anti-inflammatory activity were also substantial, $38 \pm 4\%$ of COX-2 inhibition in the aqueous extract and $85 \pm 11\%$ of COX-2 inhibition in the ethanolic extract. Regarding the alkenone profile, it was observed that the C37:3, C37:2, and C38:2 were the most abundant alkenones, with values exceeding 500 mg $(100\ g\ dw)^{-1}$, being the total alkenone content approximately 2,500 mg $(100\ g\ dw)^{-1}$. Finally, the bioaccessibility of studied compounds and bioactivities was always low or even inexistent/undetected. Future work should aim at preparing extracts for nutraceutical applications or microalgal processing through decoction (tisane) for achieving higher bioaccessibility levels.

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Data availability Data will be made available on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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