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# Defatted *Nannochloropsis oculata* biomass – Waste or resource?

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ABSTRACT

Microalgae are recognized as a valuable source of a panoply of compounds. In addition to the extensively investigated lipid fraction comprising polyunsaturated fatty acids (PUFA), microalgae biomass also encompasses other compounds with potentially relevant biological activities. This work innovatively repurposes the defatted biomass (DB) of *Nannochloropsis oculata*, highlighting its potential value beyond the lipid fraction. By subjecting the DB to enzymatic hydrolysis, we explored an underutilized resource, potentially reducing waste and promoting sustainable bioprocessing. The resulting soluble fraction was chemically characterized and a comprehensive assessment of its chemical and biological activities was performed to ascertain its potential applications. Those included antioxidant, anti-hypertensive, and antidiabetic capacities, as well as potential metabolic inhibition, anti-inflammatory, and antimicrobial activities. The chemical characterization revealed the presence of several low molecular weight peptides (<1.2 kDa), as well as polysaccharides. The DB exhibited a relevant antioxidant capacity of 3.15 $\mu mol_{Trolox\ equivalent(TE)}/mg_{freeze\text{-}dried\ biomass(FDB)}$ and an $IC_{50}$ of 77.3 ± 0.3 $\mu g_{protein}$/mL concerning angiotensin-I converting enzyme inhibitory activity, while α-glucosidase activity was inhibited by 19.4 %. Biological activities revealed no relevant inhibition of metabolic activity, an immunosuppression potential and anti-inflammatory activity (decreased expression of all pro-inflammatory cytokines in lipopolysaccharide (LPS)-induced inflammation) was also observed. Moreover, significant antimicrobial activity was observed, particularly against Gram-positive bacteria. These results underscored the potential of the DB to be utilized within a biorefinery concept, thereby transforming it into a resource (co-product) rather than considering it as waste. This study is groundbreaking due to its integrative approach, being the first to report the potential bioactivities of non-lipid extracts from *N. oculata* cultivated under modulated stress conditions.

## 1. Introduction

The lipid (lipophilic) fraction of *Nannochloropsis* spp. has garnered considerable interest due to its high content of PUFA, particularly EPA. Hence, large quantities of microalgal biomass are discarded as waste after lipid extraction. Nonetheless, the residual biomass encompasses proteins, peptides, and polysaccharides, all of which have been ascertained to harbor significant bioactive properties including antioxidant, antihypertensive, and immunostimulatory [1–4]. Moreover, peptides derived from the enzymatic hydrolysis of *Nannochloropsis* spp. proteins have evidenced notable bioactive potential [5,6]. Remarkably, enzymatic hydrolysates of defatted *Nannochloropsis* biomass have demonstrated oxygen radical absorbance capacity, radical cation and radical scavenging activities [2,7]. These antioxidant properties hold substantial significance due to their pivotal role in ameliorating oxidative stress, a factor intricately linked to the pathogenesis of numerous non-communicable chronic diseases such as cancer, cardiovascular diseases, and neurodegenerative disorders. The peptides present in the microalga biomass exert their antioxidant effects through free radical scavenging and metal ion chelation, thereby reducing oxidative damage to cells and tissues [7,8].

Moreover, microalgae-derived low molecular weight peptides have

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demonstrated capability to effectively inhibit Angiotensin-1-converting enzyme (ACE-I), both in vitro [5,9] and in vivo [9,10], which holds significant therapeutic importance in the management of hypertension. The mechanisms underlying microalgal peptide-induced antihypertensive effect comprise competitive (direct antagonism) or noncompetitive (enzyme activity modulation) inhibition [4].

This dual functionality (antioxidant and antihypertensive) highlights the therapeutic potential of *Nannochloropsis* peptides in promoting cardiovascular health and preempting associated chronic diseases.

Furthermore, *N. oculata* is also notable for the biosynthesis of antimicrobial peptides, which exhibit activity against Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* [11]. These peptides demonstrate a crucial role in addressing antibiotic resistance and are recognized as promising options for medical applications, aquaculture, and the food industry [12,13]. The antimicrobial activity of microalgae peptides is frequently attributed to their capacity to disrupt bacterial cell membranes, ultimately resulting in cell lysis. The presence of specific amino acid sequences and structural features, such as alpha helical structures, reinforces their interaction with microbial membranes [12,14].

Additionally, polysaccharides have been associated with immunostimulatory activity [3] and have demonstrated anticancer, anticholinesterase, antioxidant, and antimicrobial activities [15]. Microalgal polysaccharides (including sulfated polysaccharides, exocellular polysaccharides (EPS), β-glucans, mannans or rhamnans) may modulate inflammatory pathways through various mechanisms: (i) downregulation of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and enzymes (COX-2 and iNOS) often via the inhibition of the NF-κB signaling pathway [16], or (ii) inhibition of the expression of adhesion molecules on leukocytes (selectins and integrins) which are essential for leukocyte adhesion and transmigration through the endothelium [17]. The combined effects of reducing oxidative stress, inhibiting key inflammatory mediators, and modulating immune cell activity underscore the potential of microalgal polysaccharides as therapeutic agents for managing chronic inflammatory conditions.

In light of the diverse range of compounds extractable from *Nannochloropsis* spp. biomass, an integrated biorefinery approach may be implemented in its cultivation. This approach presents the potential to optimize the output by improving the yield of high-value compounds [1,2], which may subsequently be utilized for the development of novel products within the cosmetics, food, or pharmaceutical industries.

The purpose of the current work, as outlined in Fig. 1, was to harness the full potential of *N. oculata* defatted biomass (DB), conventionally considered disposable (waste) once the lipid fraction is extracted, by comprehensively exploring its health-promoting potential. Hence, the DB was submitted to enzymatic hydrolysis, subsequent to which the soluble fraction of the hydrolysate (SFH) was characterized in relation to its protein/peptide and carbohydrate contents. Following this, a thorough investigation of the antioxidant, anti-hypertensive, antidiabetic, anti-inflammatory, and antimicrobial activities was conducted, and correlations were tentatively established with the specific SFH composition.

The study hypothesizes that those SFH compounds possess significant bioactive properties that can be leveraged to address pressing health issues, promote sustainable practices, and contribute to scientific advancements in the fields of nutrition and medicine.

To the best of our knowledge, this investigation represents the first comprehensive analysis of the bioactivities of non-lipidic extracts derived from *N. oculata* cultivated under modulated stress conditions. This study provides an in-depth and integrated perspective on the potential health benefits of these extracts, thereby expanding the current understanding of the bioactive potential of microalgal components beyond their lipid content.

## 2. Materials and methods

### 2.1. Reagents

Phosphate buffer saline (PBS), sodium azide ($NaN_3$), zinc chloride ($ZnCl_2$), trifluoroacetic acid, 2-Deoxyglucose, methylimidazole, dimethyl sulfoxide (DMSO), $CH_3I$, $NaBD_4$, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), fluorescein, 2,2′-azobis(2-methylpropionamidine) dihydrochloride, peptidyl-dipeptidase A (EC 3.4.15.1), α-glucosidase, *p*-nitrophenyl-α-D-glucopyranoside, acarbose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium alginate, α-amylase, pepsin, hydrochloric acid, porcine

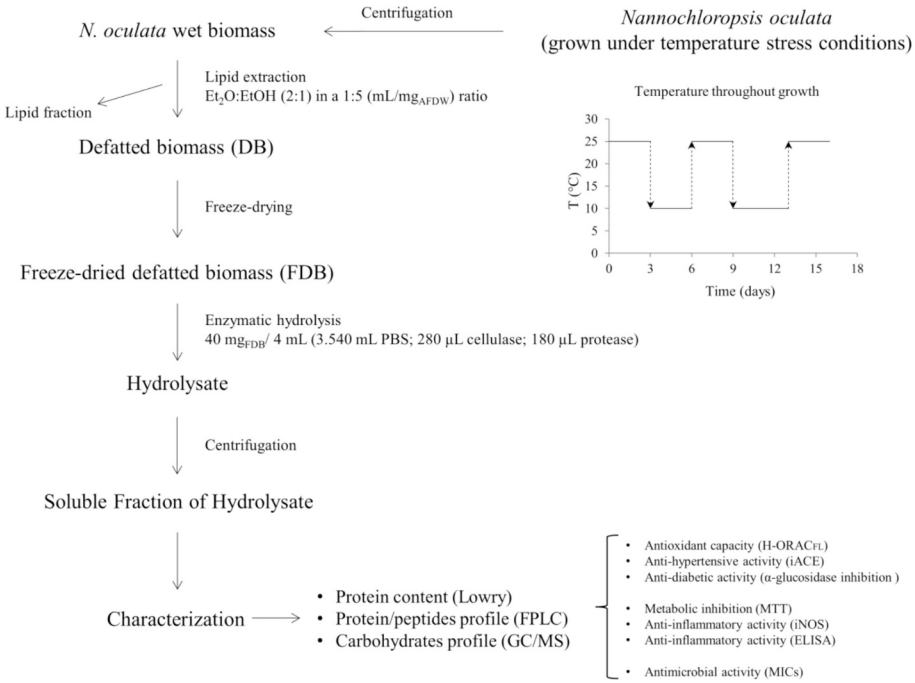


**Fig. 1.** Outline of the study, from culture growth to soluble fraction of hydrolysate (SFH) characterization and activities (chemical and biological) assessment.

pancreatin, bile salts, and Griess reagent were from Sigma Aldrich (St. Louis, MO, USA).

Intramolecularly quenched fluorescent tripeptide o-aminobenzoylglycyl-*p*-nitrophenylalanylproline (Abz-Gly-Phe($NO_2$)–Pro) was acquired from Bachem Feinchemikalien (Bubendorf, Switzerland), fetal bovine serum from Biowest (FBS; Nuaillé, France), penicillin-streptomycin-fungizone from Lonza (Basel, Switzerland), and calf bovine serum iron-fortified were obtained from ATCC (Manassas, VA, USA).

Diethyl ether ($Et_2O$), methanol (MeOH), chloroform ($CHCl_3$), sodium borohydride, and TrypLE Express were purchased from Thermo Scientific (Waltham, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM), non-essential amino acids (NEAA) and L-glutamine without pyruvate were obtained from Gibco, Thermo Scientific (Waltham, MA, USA), and lipopolysaccharides from *Escherichia coli* O111:B4 (LPS) from Invitrogen, Thermo Scientific (Waltham, MA, USA).

Ethanol (EtOH) and sodium chloride (NaCl) were from Honeywell (Charlotte, NC, USA), while glycerol, acetic anhydride and sodium hydroxide (NaOH) were from PanReac (Barcelona, Spain). Celusoft Supreme and NewPro 16 L were acquired from Novozymes (Frederiskberg, Denmark), while standard proteins were from GE Healthcare (Milwaukee, WI, USA).

### 2.2. Microalga and growth conditions

*Nannochloropsis oculata* CCAP 849/1 was grown in modified artificial seawater medium (ASW) [18] in 250 mL Erlenmeyer flasks, with a working volume of 100 mL. The microalga was grown for 16 days in a climate chamber S600PL (Aralab, Rio de Mouro, Portugal) under a continuous light intensity of 75 μmol photons/$m^2$/s (supplied by cool daylight fluorescent lamps; Lumilux L18W/865, OSRAM, Munich, Germany), and the temperature was modulated throughout growth (Fig. 1) to stress the cells in a modulated fashion, thus increasing the EPA concentration without decreasing cell growth, according to the method described by Sousa et al., 2022 [19].

### 2.3. Defatting

*Nannochloropsis oculata* fresh biomass was collected by centrifugation at 1400*g* and 4 °C, for 5 min. Supernatant (growth medium) was then discarded and the pellet was defatted (lipid extraction; Fig. 1). Briefly, the extraction solvent ($Et_2O$:EtOH, 2:1) was added to the wet biomass (pellet) in a 1:5 (mL/mg $_{\text{ash-free dry weight (AFDW)}}$) ratio and the mixture was placed at 40 °C in a water bath for 15 min. Afterwards, the lipophilic and hydrophilic fractions were separated through solvent partition, performed by addition of water at a 2:1 ratio, vortexing and centrifuging. The lipophilic (fat containing) upper layer was then removed, and the pellet corresponding to DB was frozen at −80 °C and freeze-dried.

### 2.4. Enzymatic hydrolysis

The freeze-dried defatted biomass (FDB) was submitted to enzymatic hydrolysis (Fig. 1), which was performed by adding 3.540 mL of phosphate buffer to 40 mg of FDB and adjusting pH to 7.5, then adding 280 μL of cellulase (Celusoft Supreme) and the mixture was incubated at 53 °C for 3 h, at 150 rpm (orbital shaker). Then, 180 μL of protease (NewPro 16 L) were added and incubated at 53 °C for 6 h, at 150 rpm (orbital shaker). Afterwards, the mixture was heated to 80 °C for 10 min for enzyme inactivation and, after subsequent centrifugation, the supernatant (henceforth designated SFH, which contained the proteins/peptides and polysaccharides) was collected and stored at −20 °C for further analyses.

### 2.5. Soluble fraction of hydrolysate characterization

#### 2.5.1. Protein and peptides

Initially, protein quantification was performed according to Lowry et al., 1951 [20] and, subsequently, the molecular mass distribution of the protein components was analyzed by size exclusion chromatography using Fast Protein Liquid Chromatography (FPLC). The chromatographic runs were performed at a flow of 0.5 mL/min with 25 mM phosphate buffer (pH 7), containing 150 mM NaCl and 0.2 g/L $NaN_3$. Standard proteins with known molecular masses (Thyroglobulin, 669 kDa; Ferritin, 440 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa; Ribonuclease A, 13.7 kDa; Aprotin, 6, 5 kDa; a synthetic peptide of 1.2 kDa; Tryptophan, 204 Da) were used to calibrate the system. The AKTA pure 25 L system (GE Healthcare Life Sciences, Germany) was used in a configuration consisting of two high-performance piston pump systems, a pressure monitoring system for column protection, a mixing chamber, a V9-IA injection valve, a Superdex® 200 10/300 GL column connected in series to a Superdex Peptide 10/300 GL column (GE Healthcare Life Sciences, Munich, Germany), and a length U9-L UV detector fixed wave at 280 nm. The system was controlled by UNICORN software.

#### 2.5.2. Carbohydrates

*2.5.2.1. Neutral sugar analysis.* Neutral sugars were determined as alditol acetates by gas chromatography. A hydrolysis with 2 M trifluoroacetic acid for 1 h at 120 °C was performed. 2-Deoxyglucose was used as internal standard. Monosaccharides were reduced with sodium borohydride and acetylated by acetic anhydride using methylimidazole as catalyst. The alditol acetate derivatives formed were analyzed by gas chromatography-flame ionization detector (GC-FID) equipped with a 30 m column DB-225 (J&W Scientific, USA) with i.d. and film thickness of 0.25 mm and 0.15 μm, respectively. The oven temperature program used was: initial temperature 200 °C, a rise in temperature at a rate of 40 °C/min until 220 °C, standing for 7 min, followed by a rate of 20 °C/min until 230 °C and maintaining this temperature 1 min. The injector and detector temperatures were, respectively, 220 and 230 °C. The flow rate of the carrier gas ($H_2$) was set at 1.7 mL/min. The hydrolysis and analysis of the samples was performed in triplicate. The monosaccharides identification was performed by comparison with the standards retention time.

*2.5.2.2. Methylation analysis.* Linkage analysis was carried out by methylation as described by Ciucanu and Kerek, 1984 [21] and adapted by Coimbra et al., 1996 [22]. The sample was dissolved in 1 mL of anhydrous dimethylsulfoxide (DMSO), then powdered NaOH (40 mg) was added and allowed to react during 30 min, followed by addition of $CH_3I$ (80 μL), which was allowed to react during 20 min. Then, 80 μL of $CH_3I$ were added and allowed to react for 20 min. The methylated material was dissolved in 1:1 (v/v) $CHCl_3$:MeOH and the solution was dialyzed against 50 % EtOH and evaporated. The methylated polysaccharides were hydrolyzed with 2 M TFA at 121 °C for 1 h, reduced by $NaBD_4$ and acetylated as previously described for neutral sugar analysis.

The partially methylated alditol acetates were separated and analyzed by gas chromatography–mass spectrometry on an Agilent Technologies 6890N Network. The gas chromatograph was equipped with a DB-1 (J&W Scientific) capillary column (30 m length, 0.25 mm of internal diameter and 0.15 μm of film thickness). The samples were injected in splitless mode (time of splitless 5 min) with the injector operating at 220 °C, and using the following temperature program: 45 °C for 5 min with a linear increase of 10 °C/min up 140 °C, and standing for 5 min at this temperature, followed by linear increase of 0.5 °C/min up to 170 °C, and standing for 1 min at this temperature, followed by linear increase of 15 °C/min up to 280 °C, with further 5 min at 280 °C. The helium carrier gas had a flow rate of 1.7 mL/min and

a column head pressure of 2.8 psi. The gas chromatograph was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range *m/z* 40–500 in a 1 s cycle in a full scan mode acquisition.

### 2.6. In vitro assays

#### 2.6.1. Biochemical assays

*2.6.1.1. Oxygen radical absorbance capacity (H-ORAC$_{FL}$).* Antioxidant capacity was determined using an oxygen radical absorbance capacity ($ORAC_{FL}$) assay, according to the method of Poyato et al., 2013, with slight changes [23]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) stock solution (1 mM) was prepared in a phosphate buffer solution (PBS; 75 mM, pH 7.4) and stored at −20 °C. When the assays were performed, an initial Trolox solution of 100 μM was prepared, and further diluted, with PBS to obtain standards from 10 to 80 μM. PBS was also used to dilute samples and as a blank.

The assays were performed in 96-well black microplates (Thermo Scientific™, Nunc™, Denmark), with 20 μL of sample (standards or blank), to which 120 μL of fluorescein (116.66 nM) were added, and equilibrated for 10 min at 37 °C. Afterwards, the reaction was initiated by the addition of 60 μL of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (48 mM), and immediately read (Synergy H1, Biotek Instruments, Winooski, VT, USA), throughout 80 min, at 1 min intervals. The results were expressed as $\mu mol_{Trolox\ equivalent\ (TE)}/mg_{FDB}$.

*2.6.1.2. Angiotensin-I converting enzyme inhibitory activity (iACE).* Angiotensin-I converting enzyme (ACE) inhibitory activity was determined according to the method described by Sentandreu and Toldrá, 2006, with slight modifications [24]. Briefly, an ACE stock solution was prepared by dissolving the commercial enzyme ACE (peptidyl-dipeptidase A, EC 3.4.15.1) in a solution of 50 % glycerol in ultrapure water, to obtain a final concentration of 1 U/mL. The solution was aliquoted and stored at −20 °C, and a working solution was prepared daily by diluting the stock solution 1/24 (to 42 mU/mL), in 0.1 μM $ZnCl_2$ and 0.15 M Tris buffer, pH 8.3. The substrate (intramolecularly quenched fluorescent tripeptide o-aminobenzoylglycyl-*p*-nitrophenylalanylproline (Abz-Gly-Phe($NO_2$)–Pro)) (0.45 mM) was prepared in 1.125 M NaCl and 0.15 M Tris buffer, pH 8.3.

Initially, 40 μL of ACE working solution (control and sample) or ultrapure water (blank and sample blank) were added to each well, followed by 40 μL of ultrapure water (blank and control) or sample (sample and sample blank). The sample blank was performed with non-diluted, or 1/2 diluted, samples. The enzymatic reaction was started by addition of 160 μL of substrate, and mixture was incubated for 30 min at 37 °C. Generally, dilutions from 1/1 to 1/32 were performed to determine the $IC_{50}$, which is defined as the concentration of the inhibitor needed to inhibit 50 % of the ACE activity. Assays were performed in black 96-well microplates, and read in a Synergy H1 microplate reader, using excitation and emission wavelengths of 350 and 420 nm, respectively. Inhibitory activity was expressed as percentage of inhibition of maximum ACE activity (observed in control), through Eq. (1):

$$iACE\ (\%) = \left(\left(F_{control} - F_{blank}\right) - \left(F_{sample} - F_{sample\ blank}\right)\right) \times \frac{100}{F_{control} - F_{blank}} \quad (1)$$

*2.6.1.3. α-Glucosidase inhibitory activity.* The α-glucosidase inhibitory activity assay was performed according to Kwon et al., 2008, methodology [25]. Each 50 μL extract, prepared at a concentration of 10 mg of lyophilized solids per mL, was combined with 100 μL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase enzyme solution (1.0 U/mL). This mixture was then pre-incubated at 25 °C for 10 min. Subsequently, 50 μL of a 5 mM *p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) were added to each well at 5-s intervals. The reaction mixtures were incubated at 25 °C for an additional 5 min, during which the absorbance of each individual well was measured at 405 nm using a Synergy H1 microplate reader. The results were compared to a control consisting of 50 μL of the buffer solution in lieu of the extract. Acarbose at a concentration of 10 mg/mL served as a positive control. The α-glucosidase inhibitory activity was expressed as inhibition percentage, calculated using Eq. (2):

$$\alpha - Glucosidase\ inhibition\ (\%) = \frac{\left(\Delta Abs_{control} - \Delta Abs_{sample}\right)}{\Delta Abs_{control}} \times 100 \quad (2)$$

#### 2.6.2. Cell-based assays

*2.6.2.1. Cell lines.* Human Caucasian n adenocarcinoma epithelial cells and human colon mucous-secreting epithelial cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC, Wiltshire, United Kingdom), Caco-2 (ECACC 86010202), and HT29-MTX-E12 (ECACC 12040401), respectively, and mouse macrophages RAW 264.7 (ATCC® TIB-71™) were purchased from ATCC (Manassas, VA, USA). Cells were cultured at 37 °C in a humidified atmosphere of 95 % air and 5 % $CO_2$ as monolayers, using Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine without pyruvate, containing 10 % (v/v) of fetal bovine serum (FBS). The culture medium of Caco-2 and HT29-MTX-E12 cells was further supplemented with 1 % (v/v) of non-essential amino acids.

To perform the cell culture assays SFH was diluted in the corresponding medium to achieve concentrations within the range of 10–100 $\mu L_{SFH}$/mL, corresponding to 100–1000 $\mu g_{FDB}$/mL, for MTT metabolic inhibition assay, and 100 $\mu L_{SFH}$/mL (1000 $\mu g_{FDB}$/mL), for the remaining assays.

*2.6.2.2. Metabolic inhibition (MTT assay).* The cytotoxicity assay was conducted following ISO 10993-5 [26] guidelines, with minor adjustments/modifications, employing the MTT viable dye. In brief, cells were seeded at a density of $1.0 \times 10^5$ cells per mL into wells of 96-well tissue culture plates (Thermo Scientific, Denmark) and allowed to adhere for 24 h. Subsequently, the culture medium was replaced by 100 μL of the sample, diluted in culture medium at varying concentrations. DMSO-containing media (40 %) was utilized as the negative control, while fresh medium served as the positive control, representing cells in standard growth conditions. Following a 24-h exposure period, 100 μL of MTT solution (0.5 mg/mL) was added to each well, and the plates were incubated at 37 °C in darkness. Post-incubation for 2 h, the MTT solution was discarded and 100 μL of DMSO were added to each well to dissolve the formazan crystals. The plates were then gently shaken in a light-protected environment for 10 min, after which absorbance was measured at 570 nm using a Synergy H1 microplate reader. The percentage of metabolic inhibition was calculated using Eq. (3) and was only considered significant if it was above 30 %, according to the international standard [26].

$$Metabolic\ Inhibition\ (\%) = \frac{Abs_{positive\ control} - Abs_{sample}}{Abs_{positive\ control}} \times 100 \quad (3)$$

*2.6.2.3. Anti-inflammatory activity*

*2.6.2.3.1. Inhibition of nitric oxide production (iNOS).* Nitric oxide production was evaluated according to the method proposed by Abu-Darwish et al., 2013 [27] and Queiroz et al., 2017 [28]. Briefly, after being grown to 80–90 % confluence and detached using TrypLE Express, RAW 264.7 cells were seeded at $2.5 \times 10^5$ cells/mL in 24 wells microplates and cultured for 24 h (at 37 °C, in a humidified atmosphere of 95 % air and 5 % $CO_2$). Culture medium was then carefully replaced by fresh medium (basal control) or fresh medium containing the SFH samples (at concentrations ranging from 10 to 100 $\mu L_{SFH}$/mL). Additional media were also prepared, with 1 μg/mL of lipopolysaccharide (LPS) (to stimulate inflammation), with or without the presence of SFH

at the different concentrations. Cells were then incubated for 24 h, and afterward supernatant was collected and an equal volume of Griess reagent (0.1 % (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride, and 1 % (w/v) sulfanilamide, prepared in 5 % (w/v) $H_3PO_4$ (v/v)) was added. The mixture was left to react in the dark for 30 min, and absorbances at 550 nm were read in a Synergy H1 microplate reader. The colorimetric reaction between Griess reagent and nitrite (nitric oxide (NO) end-product) present in the supernatants allowed determining NO production, and results were expressed in NO production as a percentage of control.

*2.6.2.3.2. Cytokines expression (ELISA).* Caco-2 cells were grown to 80–90 % confluence, detached using TrypLE Express, seeded at 2.5 × $10^5$ cells/well in a 24 wells microplate and incubated for 24 h at 37 °C in a humidified atmosphere of 95 % air and 5 % $CO_2$. After 24 h, the culture medium was carefully replaced with medium supplemented with SFH samples (previously prepared to obtain 100 $\mu L_{SFH}$/mL; 1000 $\mu g_{FDB}$/mL) and the plate re-incubated for another 24 h. As an inflammation control, LPS was used at 1 μg/mL while for basal activity control plain medium was used. At the end of the assay, supernatants were collected, centrifuged to remove debris, and stored at −80 °C for further analysis. Interleukins 6 (IL-6) and 8 (IL-8) and TNF-α detection was performed by ELISA kits (Biolegend, San Diego, CA, USA) according to the manufacturers' instructions.

### 2.6.3. Antimicrobial activity

Antimicrobial activity was assessed through minimum inhibitory concentrations (MICs) which were determined, based on the microdilution method described in Wiegand et al., 2008 [29], for representatives of Gram-positive (*Listeria monocytogenes*, *Listeria innocua*, *Bacillus cereus*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA)) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium), and one yeast (*Candida albicans*).

*Bacillus cereus*, *S. aureus*, MRSA, *S. enterica* serovar Enteritidis, *S. enterica* serovar Typhimurium and *P. aeruginosa* were obtained from the American Type Culture Collection (ATCC, USA; ATCC 11778, ATCC 6538, ATCC 29213, ATCC 13076, ATCC 14028 and ATCC 10145, respectively). *Listeria innocua*, *E. coli* and MSSA were obtained from the National Collection of Type Cultures (NCTC, UK; NCTC 10528, NCTC 9001 and NCTC 8532, respectively). *Listeria monocytogenes* was a food isolate from Escola Superior de Biotecnologia's culture collection (ESB, Porto, Portugal; ESB 3562), and *Candida albicans* was obtained from the culture collection of the University of Gothenburg (CCUG, Gothenburg, Sweden; CCUG 49242).

Briefly, Gram-positive bacteria were grown in Brain Heart Infusion (BHI) broth, Gram-negative bacteria in Luria-Bertani (LB) broth, and *C. albicans* in Sabouraud Dextrose Broth (SDB), all from Biokar Diagnostics (Beauvais, France). Bacteria were incubated for 24 h at 37 °C, and *C. albicans* at 30 °C. Afterward, to prepare the inocula, cultures were centrifuged and biomass was resuspended in Muller–Hinton (MH, Biokar Diagnostics) broth, except *C. albicans* which was resuspended in SDB, to achieve an absorbance (optical density; OD) at 600 nm ($OD_{600}$) of ~1.0 (corresponding to 1 × $10^8$ colony forming units (CFU)/mL), which was then diluted 100-fold in the corresponding media (achieving 1 × $10^6$ CFU/mL). In parallel, 2-fold dilutions of SFH were prepared in a 96-well microplate, by diluting SFH in a 1:1 ration in the growth media. The previously prepared inocula were then added 1:1 (v/v) to the microplate wells containing SFH dilutions, thereby obtaining a microorganism concentration of 5 × $10^5$ CFU/mL. Microplates were incubated for 24 h at 37 or 30 °C, according to the microorganism, and minimum inhibitory concentrations (MICs) were defined as the lowest SFH concentrations for which no bacterial/yeast growth was observed with unaided eye.

### 2.7. Statistical analysis

Statistical analyzes were performed with SPSS software (IBM®, USA), utilizing Student's *t*-test for pairwise comparisons, with a statistical significance of 0.05. All assays were performed in triplicate.

## 3. Results and discussion

### 3.1. SFH characterization

#### 3.1.1. Protein and peptides

Peptides are small molecules which have been described as having the potential to exert several biological activities, such as antihypertensive, antidiabetic and antioxidant, among others. However, to be active, microalgae peptides need to be released from proteins, which may be achieved through hydrolysis [30]. In this sense, to increase the biological activity potential of the *N. oculata* DB, FDB was enzymatically hydrolyzed with a protease, together with a cellulase. After separation of the SFH from the hydrolyzed biomass, analysis of its protein content (soluble protein) revealed a concentration of 4.00 ± 0.01 $mg_{protein}/mL_{SFH}$, which corresponded to 0.40 $mg_{protein}/mg_{FDB}$ (40 %). This is in accordance with what was reported by Qian et al., 2013 [31], that determined a 38.6 % protein content in *N. oculata* biomass after lipid extraction. Norzagaray-Valenzuela et al., 2017 also determined the composition of *Nannochloropsis* sp. residual biomass, which was found to comprise 25.5 % of protein [2].

After determination of the protein content, FPLC analysis was performed, which allowed to determine the protein/peptide profile of the SFH. Results unveiled the presence of a large amount of several peptides (peaks below 1.2 kDa), as well as proteins with 669, 158, 75, 43, 29 and 13.7 kDa (Fig. 2).

The presence of peptides with low molecular weight is important when bioactivity is concerned, since molecular weight has an impact in their transport route within the organism. The passage through the intestinal barrier is partly conditioned by the weight of the peptide and, although both high and low molecular weight peptides can cross it, the probability increases with the decrease of the molecular weight. Hence, low molecular weight peptides are more prone to pass the intestinal barrier and, therefore, to be able to impact a biological activity [32].

#### 3.1.2. Carbohydrate analysis

The total sugars content of the SFH determined by GC-FID after the acidic hydrolysis was 4.4 %, (w/w), comprising mannose (Man; 47 mol %), galactose (Gal; 29 mol%), glucose (Glc; 20 mol%), and ribose (Rib; 4 mol%). This composition was in agreement with that reported by

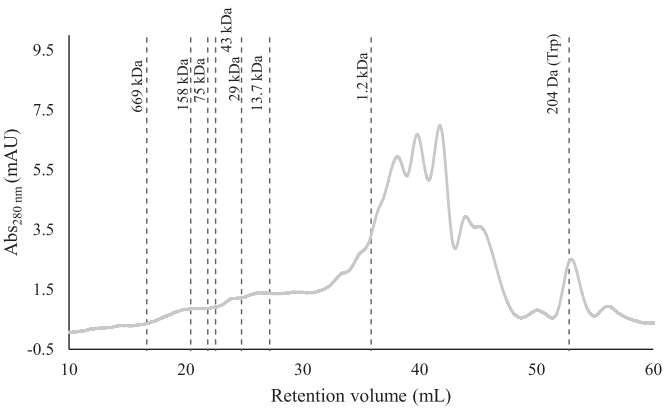


**Fig. 2.** Molecular weight distribution of SFH obtained by FPLC (Thyroglobulin, 669 kDa; Aldolase, 158 kDa; Conalbumin, 75 kDa; Ovalbumin, 43 kDa; Carbonic anhydrase, 29 kDa; Ribonuclease A, 13.7 kDa; synthetic peptide of 1.2 kDa; Tryptophan, 204 Da).

Pandeirada et al., 2019 for water-soluble polysaccharides of *N. oculata* [3].

The glycosidic-linkage analysis (Table 1) of the SFH revealed the presence of terminal (t-), (1 → 4)-, (1 → 3)-, and (1 → 4,6)-linked Man residues. These residues could be related with the presence of (α1 → 3)-, (α1 → 4)-mannans, as reported by Pandeirada et al., 2019 [3]. Glucose residues occur mainly as terminal, (1 → 3)-, (1 → 4)-, and (1 → 3,6)-linked residues, which could be due to the presence of mixed-linked (β1 → 3, β1 → 4)-glucans [3]. Galactose was observed as (1 → 2)- and (1 → 2,6)-linked residues, which have also been reported to be present in the *N. oculata* polysaccharides composition [3]. These polysaccharides revealed immunostimulatory potential for B lymphocytes in vitro and can be considered dietary fibres with prebiotic activity.

### 3.2. In vitro assays

The selection of these bioactivities is based on their significant impact on health and their potential therapeutic applications. By investigating the antioxidant, anti-hypertensive, antidiabetic, anti-inflammatory, and antimicrobial properties of *N. oculata*-derived peptides and polysaccharides, this study aims to harness the full potential of defatted microalgal biomass, contributing to the development of novel functional foods, nutraceuticals, and pharmaceuticals.

#### 3.2.1. Biochemical assays

*3.2.1.1. Antioxidant capacity, anti-hypertensive and antidiabetic activities.* The potential toxicity associated with synthetic antioxidants paved the way for the quest for safe, natural alternatives for neutralizing free radicals and preventing related diseases. Consequently, there has been a significant surge in research exploring the capacity of microalgae-derived bioactive compounds to mitigate oxidative stress.

Antioxidant potential of the FDB was, as previously mentioned, assessed through H-ORAC$_{FL}$ assay, which showed an oxygen radical absorbance capacity of 3.15 ± 0.08 μmol$_{Trolox\ equivalent(TE)}$/mg$_{FDB}$ (Table 2). The antioxidant capacity may be partially ascribed to the peptide content, since other studies such as Medina et al., 2015 [33] and Norzagaray-Valenzuela et al., 2017 [2], have also reported antioxidant capacities of protein hydrolysates of *Nannochloropsis*. Medina et al., 2015 [33] reported an EC$_{50}$ of 0.104 μmol$_{TE}$/mL protein hydrolysate from *N. gaditana*, and an antioxidant capacity of 2.95 μmol$_{TE}$/mg$_{protein\ hydrolysate}$, similar to that reported herein, was found regarding a hydrolysate from *Nannochloropsis* sp. residual (defatted) biomass [2,33]. Moreover, peptides have been described to possess higher antioxidant capacity than the larger molecular weight proteins [6], which confers added value to SFH due to the previously mentioned higher peptide content.

The low molecular weight of these peptides constitutes a fundamental factor that substantiates their superior antioxidant activity [9,34,35]. In particular, this feature allows them to diffuse through cell membranes more efficiently and reach target sites harboring free radicals [36–38]. This higher accessibility enhances their ability as electron or hydrogen atom donors to effectively scavenge free radicals (peroxyl). Moreover, short chain peptides exhibit higher reactivity due to reduced steric hindrance and the presence of an active core mainly composed of hydrophobic amino acid residues [39–41]. This structural composition allows for efficient interaction with and neutralization of radical species, thereby enhancing their antioxidant efficacy [39].

Although numerous potential bioactivities of microalgal peptides are documented in the literature, their antioxidant potential emerges as the most promising target for further investigation. Moreover, the antioxidant potential of microalgal peptides is often considered higher than that of polysaccharides due to their ability to engage in multiple antioxidant mechanisms. Nonetheless, both peptides and polysaccharides [42] contribute to the overall antioxidant capacity of microalgae. The chemical structure of *N. oculata* polysaccharides (namely molecular weight, monosaccharide composition and sulfation degree) is determinant for the microalgae antioxidant activity, since the presence of sulfate groups improve their ability to scavenge free radicals, while the monomers provide functional groups that can interact with and neutralize reactive oxygen species [15,43,44]. The mechanisms underlying the antioxidant activity of microalgal polysaccharides encompass ROS scavenging, metal chelation, and inhibition of lipid peroxidation, with their efficacy often correlated to their sulfate content [42,45,46].

The inhibitory activity of SFH on ACE (anti-hypertensive activity), evaluated by the concentration needed to inhibit 50 % of the enzyme activity (IC$_{50}$), was determined to assess the corresponding anti-hypertensive potential. Results showed that 77.3 ± 0.3 μg$_{protein}$/mL were required to inhibit 50 % (IC$_{50}$) of the ACE activity (Table 2). With a similar objective to that explored in the present work, Qian et al., 2013 studied the ACE inhibitory activity of enzymatic hydrolysates of *N. oculata* biomass after extraction of lipid fraction for biodiesel purposes [31]. The authors found that the hydrolysate with the highest activity was the one resultant from alcalase hydrolysis, which presented an IC$_{50}$ of 126 μg/mL, which is 1.6-fold higher than the obtained in the present work. The IC$_{50}$ value determined herein is also 4.8-fold lower than the one determined by Hayes et al., 2022 for a permeate fraction of *N. oculata* protein hydrolyzed with xylanase (IC$_{50}$ = 370 μg$_{protein}$/mL) [6]. Previously, Samarakoon et al., 2013 also assessed the ACE IC$_{50}$ of several *N. oculata* hydrolysates, obtained with distinct enzymes [5]. The authors found IC$_{50}$ values ranging from 2.81 to 3.37 mg/mL (when using pepsin and papain, respectively), which are over 35-fold higher than the determined herein.

The positive SFH ACE inhibitory activity may be attributed to the peptide content, since anti-hypertensive activity has been described to be associated with low molecular weight (<1 kDa) peptides [47], which were previously shown, by FPLC analysis, to be present. In fact, the low peptides size, short amino acid chains and amino acids composition are pivotal structural features which enhance the peptides' ability to interact effectively (via hydrogen bonds, electrostatic forces, hydrophobic interactions, and van der Waals forces) with the ACE non-active sites [4,35,48]. Hence, SFH may positively impact human health due to

**Table 1**
Glycosidic-linkage analysis (mol%).

| | | |
|---|---|---|
| t-Rib | 3.6 | |
| ***Total Rib*** | | ***3.6*** |
| t-Man | 4.4 | |
| 4-Man | 24.6 | |
| 3-Man | 21.8 | |
| 4,6-Man | 2.1 | |
| ***Total Man*** | | ***52.9*** |
| 2-Gal | 15.4 | |
| 2,6-Gal | 13.3 | |
| ***Total Gal*** | | ***28.7*** |
| t-Glc | 4.1 | |
| 3-Glc | 2.8 | |
| 4-Glc | 6.8 | |
| 3,6-Glc | 1.2 | |
| ***Total Glc*** | | ***14.9*** |

Rib – ribose; Man – mannose; Gal – galactose; Glc - glucose.

**Table 2**
Characterization of SFH/FDB chemical properties.

| Capacity/ activity | Assay | Result | |
|---|---|---|---|
| Antioxidant | H-ORAC$_{FL}$ | 3.15 ± 0.08 | μmol$_{Trolox\ equivalent}$/ mg$_{FDB}$ |
| Anti-hypertensive | iACE (IC$_{50}$) | 77.3 ± 0.3 | μg$_{protein}$/mL |
| Antidiabetic | α-Glucosidase inhibition | 19.4 ± 0.6 | % |

Note - results are expressed as average ± standard deviation (n = 3).

its anti-hypertensive potential demonstrated by the low $IC_{50}$ value, thereby constituting a suitable alternative to the available antihypertensive drugs which possess substantial side effects. The peptides originating from the *Nannochloropsis* genus have been mostly reported to possess a noncompetitive inhibition pattern, modulating enzyme activity through allosteric sites, inducing a conformational alteration of ACE, which either decreases its catalytic efficiency or completely impedes substrate access to the active site, irrespective of the substrate concentration [4,48]. Hence, the inhibition of the enzymatic conversion of angiotensin I to angiotensin II impedes the formation of the latter, which is a potent vasoconstrictor, leading to vasodilation and a consequent reduction in blood pressure.

Soluble fraction hydrolysate was also assessed concerning α-glucosidase inhibitory activity, which translates the antidiabetic potential. Alpha-glucosidase activity was inhibited by SFH by 19.4 ± 0.6 % (Table 2), indicating that SFH has the ability to decrease the postprandial blood glucose concentration, which is significant concerning diabetes illness [49]. The potential to decrease blood glucose may result in an antidiabetic activity of SFH. In fact, the antidiabetic potential of *Nannochloropsis* microalgae has been reported by several authors. In accordance with the observed herein, Vieira et al., 2021 also reported antidiabetic activity of a *Nannochloropsis* microalga (*N. gaditana*) [49]. In that case the authors studied the inhibition of α-amylase, another enzyme which releases glucose from the diet, and determined an $IC_{50}$ of 12.69 ± 1.39 mg/mL. Antidiabetic activities of *Nannochloropsis* microalgae were also assessed in vivo in streptozotocin-induced diabetic rats [50–52]. Nacer et al., 2020 studied the impact of the administration of *N. gaditana* to those animals and found that it decreased serum glucose levels [52]. Regarding *N. oculata*, Aboulthana et al., 2018 [50] observed, similarly to Vieira et al., 2021 [49], a decrease of α-amylase in diabetic rats fed with the microalga, as well as a decrease in glucose levels. In Nasirian et al., 2019 study, in accordance with those findings, the authors also reported a decrease in glucose serum concentration in diabetic rats which were fed with *N. oculata* [51]. These studies did not delve into the potential mechanisms supporting such glucose lowering activity, but it is believed that a competitive inhibition is involved. Although, in a different organism, it has been shown thatlow molecular weight peptides obtained from *Spirulina platensis* displayed antidiabetic activity via inhibition of α-glucosidase through binding into the active pocket of the enzyme (according to molecular docking) [53]. Most of the potential binding amino acid residues were hydrophobic (leucine, isoleucine, glycine, and tryptophan) enhancing the peptide's ability to interact to the enzyme's hydrophobic pocket, thereby stabilizing the peptide-enzyme complex. By preventing the substrate from accessing the enzymatic active site, the microalgal peptides slow the digestion of complex carbohydrates, consequently delaying the absorption of glucose into the bloodstream and averting the onset of hyperglycemia [54]. These peptides provide a natural alternative for addressing postprandial hyperglycemia and diabetes, effectively circumventing the prominent adverse effects associated with synthetic drugs [55].

Antidiabetic potential through inhibition of α-glucosidase has also been correlated with algal and microalgal polysaccharides [42,56,57]. Alpha-glucosidase inhibition is mostly of competitive nature [19], and the enzymes inhibition delays the hydrolysis of carbohydrates and consequent absorption of glucose [57].

### 3.2.2. Biological activities

*3.2.2.1. Metabolic activity.* The inhibition of metabolic activity of Caco-2, HT29-MTX-E12 and Raw 264.7 cells by SFH was assessed using the MTT assay. Results (Fig. 3a-c) showed that SFH, at the concentrations tested, did not inhibit the metabolic activity of any of the cell types assessed above the threshold considered by ISO 10993-5:2009 [26] to be significant (30 %, above which cytotoxicity is considered), except for

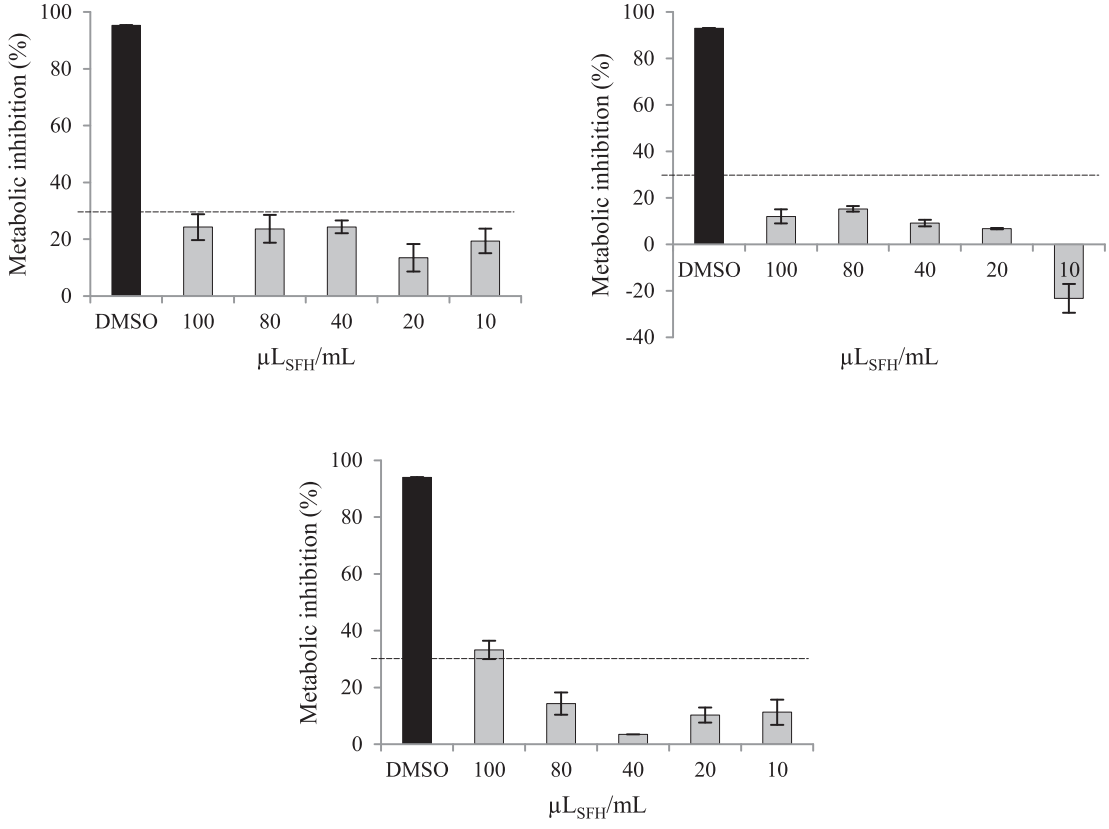


**Fig. 3.** Inhibition of metabolic activity of Caco-2 (a), Raw 264.7 (b) and HT29-MTX-E12 (c) cell lines. Results are expressed as average ± standard deviation ($n = 3$). Dashed line represents the threshold (30 %) above which metabolic inhibition is considered significant, and DMSO was utilized as positive control.

one particular case. An inhibition of metabolic activity of 33.2 ± 0.2 % was observed in HT29-MTX-E12 cells at the highest SFH concentration (100 μL/mL; 1000 $\mu g_{FDB}$/mL) tested (Fig. 3c), which was, however, only very slightly above the mentioned threshold. Furthermore, in Raw 264.7 cells with SFH at 10 μL/mL a slight increase in metabolic activity was observed (Fig. 3b), translated by negative percentage (−23.3 ± 6.2 %), which is indicative of a stimulation of the cells. In accordance with these findings, the absence of metabolic inhibition of human umbilical vein endothelial cells was also observed by Samarakoon et al., 2013, concerning a pepsin hydrolysate of *N. oculata* and purified peptides, at concentrations up to 100 μg/mL [5]. These results validated the feasibility of the utilization of the cell lines in further assays, since the results would not be affected by metabolic alterations. Accordingly, it was also demonstrated that SFH may be safely used within the range of concentrations tested to develop products intended for human consumption.

*3.2.2.2. Anti-inflammatory activity.* Macrophages are vital players in the anti-inflammatory process, which can be activated as response to pathogen presence, particularly some of its structures (e.g., LPS), and during the pathogen invasion process, NO, which is an inflammatory mediator, is secreted by immunocytes [58]. In this assay, the production of NO by LPS-stimulated Raw 264.7 cells (macrophages) was measured by the accumulation of nitrite in cell supernatants, using a colorimetric reaction with Griess reagent [27].

The SFH showed a high potential to inhibit the NO production by LPS-stimulated Raw 264.7 cells (Fig. 4) at concentrations as low as 10 μL/mL (100 $\mu g_{FDB}$/mL). All the concentrations tested were able to decrease the concentration of NO produced to <10 % of the control. Moreover, as it was previously demonstrated by the MTT assay, the presence of SFH at 10 μL/mL slightly stimulated the cell activity. Hence, it may be deduced that the inhibitory activity at this concentration was even higher than at the other tested concentrations, since the cells' stimulation by SFH presence did not result in an increase of NO concentration, in comparison with the remaining SFH concentrations at which the metabolism was not affected.

In order to further explore the anti-inflammatory potential of SFH, expression of pro-inflammatory cytokines (namely IL-6, IL-8 and TNF-α) by Caco-2 cells in LPS-induced inflammation, was assessed, with or without exposure to SFH. The expression of those cytokines at basal level, and when in contact with SFH, was also determined in cells without the inflammatory stimulus to evaluate the pro-inflammatory potential of SFH. Concerning the latter, results (Fig. 5c) showed that only TNF-α was slightly increased although to a significant degree ($p < 0.05$) when cells were exposed to SFH, while the expression of the remaining cytokines (IL-6 and IL-8) did not suffer significant alterations ($p > 0.05$). Regarding the LPS-induced inflammation, it was observed that the macrophages expression of all assessed pro-inflammatory cytokines was significantly decreased ($p < 0.05$). These results showed an anti-inflammatory potential of SFH since when the cells were induced to express pro-inflammatory cytokines, the presence of SFH decreased their expression.

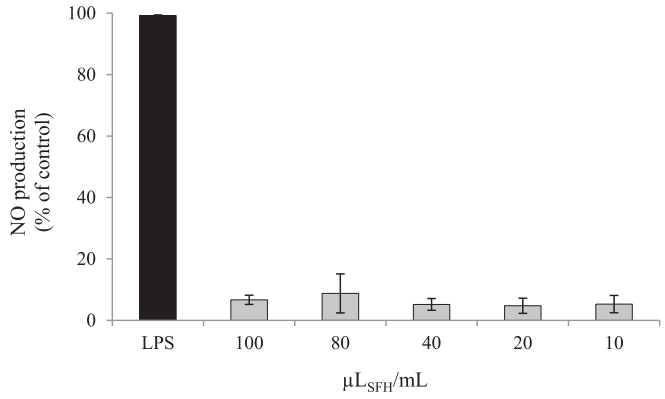


**Fig. 4.** Nitric oxide production by LPS-stimulated RAW 264.7 cells in the absence (LPS) or presence of progressively growing SFH concentrations. Results are expressed as average ± standard deviation ($n = 3$).

The anti-inflammatory activity of SFH from *N. oculata* determined herein is in accordance with previous reports concerning this bioactivity for *Nannochloropsis* microalgae.

Revianti et al., 2020 administered, by oral irrigation, a *N. oculata* solution to rats artificially infected with *Aggregatibacter actinomycetemcomitans*, the main bacterium responsible for periodontitis [59]. The study revealed a decrease of the expression of TNF-α in orally irrigated rats, concomitant with an increase in the expression of IL-10, which translated an anti-inflammatory activity of the *N. oculata* solution, when at 2.375 %. Another study, performed by Nacer et al., 2020, analyzed the impact of supplementing the diet of streptozotocin-induced diabetic rats with 10 % of *N. gaditana* [52]. As expected, in comparison with control rats, diabetic rats presented higher levels of pro-inflammatory cytokines, namely IL-6 and TNF-α. However, the authors found that the presence of microalga in the diet of the diabetic rats significantly decreased the concentration of both IL-6 and TNF-α. The anti-inflammatory activity observed herein was in accordance with the findings of both Revianti et al., 2020 [59] and Nacer et al., 2020 [52] studies.

Moreover, the immunostimulatory potential of *N. oculata* water-soluble polysaccharides was previously described by Pandeirada et al., 2019 [3], who demonstrated the enhancement of both innate and adaptive immune responses mechanisms, thereby highlighting the relevance of those compounds and their potential application.

*3.2.3. Antimicrobial activity*

Antimicrobial activities of SFH were assessed through determination of MICs against some representatives of Gram-positive and Gram-negative bacteria, and one yeast. Results (Table 3) showed that, within the range of concentrations tested (≤150 $\mu L_{SFH}/mL_{medium}$; ≤ 1.5 $mg_{FDB}/mL_{medium}$), SFH was able to inhibit the growth of three Gram-positive bacteria and one Gram-negative bacterium. Regarding the growth inhibition of Gram-positive bacteria, SFH was more active against *Listeria* species than against *S. aureus*, since lower concentrations were needed to achieve the inhibition.

The superior performance of SFH against Gram-positive bacteria, in comparison with Gram-negative bacteria, may be explained by interference of the peptides with peptidoglycan synthesis. Since the cell wall of Gram-positive bacteria possess a thicker peptidoglycan layer, the impact of disrupting its synthesis will be more pronounced in those bacteria. This is because peptidoglycan is a critical component for maintaining cell wall integrity and osmotic balance in these bacteria [60,61]. This may explain the higher number of Gram-positive bacteria for which MICs were able to be determined, as well as the lower concentration of SFH needed to achieve the antimicrobial effect. In contrast, Gram-negative bacteria possess an outer membrane that provides an additional barrier, limiting the access of antimicrobial agents to the peptidoglycan layer. This barrier makes Gram-negative bacteria inherently more resistant to many antimicrobial compounds. As a result, the concentration of SFH required to inhibit the growth of *S. enterica* serovar Typhimurium, the only Gram-negative bacterium for which a MIC was determined, needed to be the highest tested.

However, polysaccharides derived from *Nannochloropsis oculata* have also demonstrated antimicrobial activities, mainly attributed to the polysaccharides content in negatively charged sulfated heteropolysaccharides, which can interact with microbial cell membranes, leading to disruption and inhibition of bacterial growth [3,15,43]. Nonetheless, in-depth understanding of the chemical structure of polysaccharides is pivotal to precisely correlate their structural characteristics and the abovementioned biological activity [3,62].

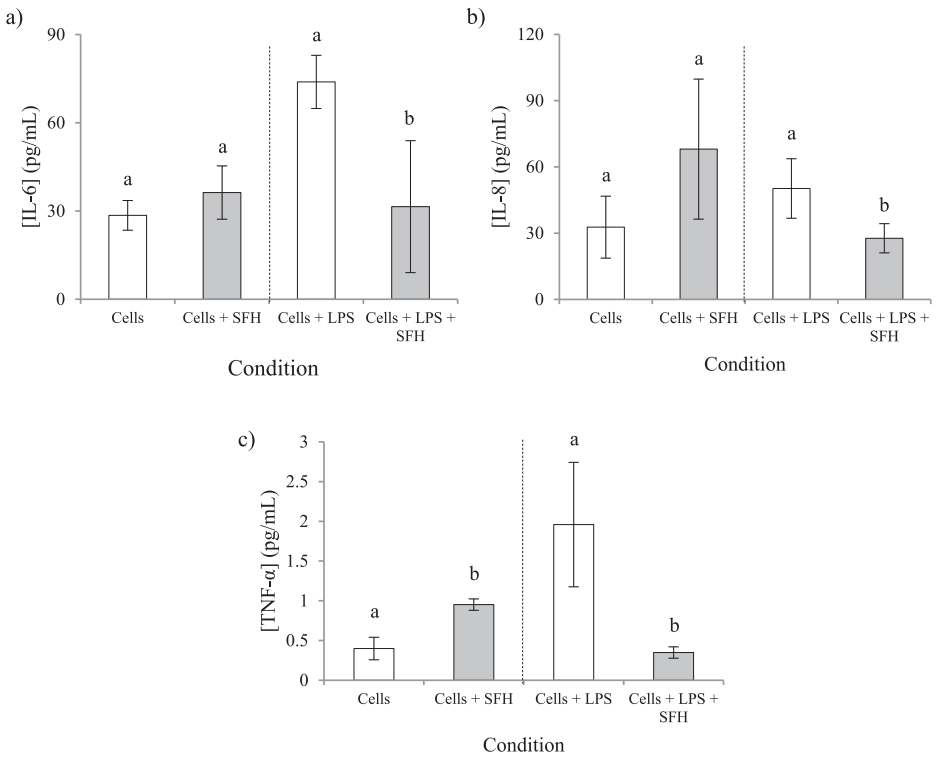


**Fig. 5.** Expression of IL-6 (a), IL-8 (b) and TNF-α (c) by Caco-2 cells in the absence (cells) or presence of SFH (cells + SFH), with (right side of the dashed line) or without (left side of the dashed line) LPS-induced inflammation. Results are expressed as average ± standard deviation ($n = 3$). Within the same condition (with or without LPS-induced inflammation), different lower-case letters indicate statistically significant difference ($p < 0.05$).

**Table 3**
Minimum inhibitory concentrations ($mg_{FDB}/mL_{medium}$) of soluble fraction of hydrolysate against different Gram-positive and Gram-negative bacteria and yeast.

| | Microorganism | MIC value ($mg_{FDB}/mL_{medium}$) |
|---|---|---|
| Gram-positive bacteria | *Listeria innocua* | 0.8 |
| | *Listeria monocytogenes* | 0.9 |
| | *Bacillus cereus* | >1.5 |
| | *Staphylococcus aureus* | 1.1 |
| | MRSA | >1.5 |
| | MSSA | >1.5 |
| Gram-negative bacteria | *Escherichia coli* | >1.5 |
| | *Pseudomonas aeruginosa* | >1.5 |
| | *Salmonella enterica* serovar Enteritidis | >1.5 |
| | *Salmonella enterica* serovar Typhimurium | 1.5 |
| Yeast | *Candida albicans* | >1.5 |

MRSA – methicillin-resistant *S. aureus*; MSSA – methicillin-susceptible *S. aureus*.

## 4. Conclusions

Overall, this comprehensive study revealed, for the first time, that defatted *N. oculata* biomass, cultivated under modulated stress conditions, is indeed a valuable resource, since after lipid extraction the microalga biomass still possesses compounds which may be further explored due to their potential chemical capacities and biological activities. From an industrial/biorefinery perspective, the value of the microalga does not end when its lipidic content is removed, but the composition of the remaining biomass, comprising peptides and polysaccharides, confers it a potential to be a valuable resource. Unlike previous studies that might focus on a single bioactivity, this research provides a thorough evaluation of antioxidant, anti-hypertensive, anti-diabetic, immunosuppressive, and anti-inflammatory activities. This multifaceted approach underscores the potential multifunctional health benefits of the defatted biomass (and, consequently, of SFH), thus providing an added value to functional foods or nutraceutical/therapeutic agents developed with such ingredient. Moreover, the immunosuppression potential and anti-inflammatory activity of SFH may prove beneficial for those suffering from autoimmune diseases, since it may ameliorate/mitigate the immune system imbalance characteristic of these pathologies, which results in the inflammation process. These findings fully corroborate our initial hypothesis regarding the bioactive potential of SFH compounds. This work is groundbreaking in its comprehensive assessment of *N. oculata* DB, shifting the paradigm from waste to resource. The study's integrative approach to characterizing and evaluating bioactivities, coupled with the potential for sustainable biorefinery practices, marks a significant advancement in utilizing microalgae biomass.

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## Author statement

All the authors agree to submit the manuscript entitled "Defatted Nannochloropsis oculata biomass – waste or resource?", for consideration for publication in Algal Research. This original research has not been published elsewhere before.

## CRediT authorship contribution statement

**Sérgio Sousa:** Writing – review & editing, Writing – original draft,

Methodology, Investigation, Formal analysis, Conceptualization. **Manuela Machado:** Writing – original draft, Investigation, Formal analysis. **Ezequiel Coscueta:** Validation, Investigation, Formal analysis. **Andreia S. Ferreira:** Investigation, Formal analysis. **Cláudia Nunes:** Validation, Methodology, Investigation, Formal analysis. **Manuel A. Coimbra:** Validation, Methodology, Funding acquisition. **Ana C. Freitas:** Validation, Supervision, Methodology, Funding acquisition, Conceptualization. **Ana P. Carvalho:** Validation, Supervision, Methodology, Funding acquisition, Conceptualization. **Ana M. Gomes:** Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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