



Secondary metabolites of cyanobacteria from Cape Verde Archipelago act as NO donors with potential application in dermatology and cosmetics[☆]

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ABSTRACT

Nitric oxide (NO) is a versatile mediator implicated in a variety of physiological processes, with the ultimate goal of maintaining organism homeostasis. In the present work, aqueous extracts from ten cyanobacteria strains isolated from the Cape Verde archipelago were explored as potential NO donors. None of the strains are toxin producers, as demonstrated by PCR and LC-MS analysis. The extracts were mainly composed by phycobiliproteins (9.1 to 43.8 %), followed by polysaccharides (7.7 to 35.1 %), phenols (1.49 to 3.3 %) and chlorophylls (0 to 0.45 %). All the extracts revealed antioxidant potential, with *Salileptolyngbya* sp. LEGE 181184 presenting the lowest IC₅₀ value for superoxide anion radical scavenging (46.50 µg mL⁻¹), and ability to inhibit the pro-inflammatory enzyme lipoxygenase (LOX), *Salileptolyngbya* sp. LEGE 181150 presenting an IC₂₅ similar to the positive control quercetin (28.49 and 31.77 µg mL⁻¹, respectively), highlighting the potential of cyanobacteria extracts as natural ingredients for LOX inhibition. All the extracts were able to increase the NO produced by the macrophage cell line RAW 264.7 through iNOS modulation (from concentrations starting in 12.5 µg mL⁻¹), in a similar mechanism and superior extend to that of LPS. None of the extracts induced cytotoxicity to RAW 264.7 cells and to the endothelial hCMEC/d3, the fibroblast 3 T3/L1 and the keratinocytes HaCaT cell lines, and no environmental hazard is predicted, as demonstrated through the zebrafish (*Danio rerio*) embryo acute toxicity test (zFET). This pioneer study points-out cyanobacteria aqueous extracts as innovative and biobased natural antimicrobial ingredients which, through a NO-donating mechanism, may potentially act against important antibiotic-resistant strains, thus being worth of consideration as therapeutic agents in dermatology.

1. Introduction

Nitric oxide (NO) is an essential pleiotropic physiological regulator, synthesized enzymatically in various tissues of the human body by nitric oxide synthases (NOS), through the conversion of L-arginine into L-citrulline and NO. There are three main isoforms of NOS: endothelial (eNOS), neuronal (nNOS) and induced (iNOS), in addition to a mitochondrial isoform (mtNOS) [1]. eNOS and nNOS are calcium-dependent enzymes that produce small amounts of NO for cellular signaling and protein regulation. In contrast, iNOS, which is calcium-independent, generates large quantities of NO in response to inflammatory signals and other stimuli [2]. NO can also be generated by non-enzymatic

processes, such as the photodecomposition of nitrite and S-nitrosothiols under UV-A radiation [3], and through the activity of commensal bacteria that possess the enzyme nitrate reductase, capable of acting on sweat-derived nitrite [2].

As mediator in homeostasis NO participates in metabolic processes, such as cardiac oxygen metabolism [4], maintenance of the vasodilator tone that is essential for the regulation of blood flow and pressure erectile response [5], insulin secretion and glucose metabolism [6]. In central nervous system, NO acts as a mediator of cell-cell signaling namely in inflammatory processes [7].

Particularly concerning the skin, NO performs vital functions, such as dermal vasodilation and consequently regulation of body

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temperature, response to infection and wound healing [8]. NO plays a crucial role in wound healing by supporting cell proliferation, collagen synthesis, angiogenesis, and both antimicrobial and inflammatory responses. Its application in dermatology extends to treatments for bacterial infections, wound healing, leishmaniasis, and cosmetic issues such as acne and skin aging [9].

Beyond natural production, research in NO focusing on advanced materials designed to release it in a controlled and sustained manner to improve therapeutic is increasing. Recent advancements emphasize the use of innovative NO-releasing nanomaterials and biomaterials, which offer targeted and sustained release of NO. This controlled delivery is particularly interesting for skin as significantly enhances the effectiveness of skin treatments [2,9], including cosmetic applications for conditions such as acne vulgaris [10], in addition to the crucial role of NO in the root hair formation [11].

Studies involving effects on the production and function of NO by microalgae and cyanobacteria extracts and compounds, have shown promise as recently described in studies where it was found that the aqueous extracts of the Cyanobacteria *Limnospira* (formerly *Arthrospira*) improved vasomotor function in the aorta of aged and hypertensive rats by enhancing antioxidant defenses and increasing NO availability [12,13].

Cyanobacteria, commonly referred to as blue-green algae, are a diverse group of photosynthetic organisms that significantly contribute to both aquatic and terrestrial ecosystems. While not all Cyanobacteria produce cyanotoxins, they are notorious for the harmful toxins they do produce, such as microcystins, cylindrospermopsins, and anatoxins, which pose significant risks to human health [14]. Despite this, some species of cyanobacteria generate high-value bioactive compounds, including pigments and specialized metabolites, that have valuable applications in the clinical, pharmaceutical, cosmetic, and textile industries [15]. These bioactive compounds, such as phycobiliproteins (PBPs), polysaccharides, carotenoids, fatty acids, and various phenolic compounds, exhibit diverse biological activities with increasing industrial interest [16]. An example, cyanobacterial PBPs, commercially produced from species like *Limnospira platensis* (formerly *Arthrospira platensis*), *Anabaena* sp., and *Galdieria sulphuraria*, have been widely used for their antioxidant, anti-inflammatory, nutraceutical and anticancer benefits [17,18].

This growing application of cyanobacterial-based ingredients extends into the realm of cosmeceuticals, where their inclusion in formulations can complement the benefits provided by other compounds. For instance, PBPs derived from *Nostoc* sp. have shown promise as natural and effective ingredients for skin care products such as soaps, anti-acne gels and hand sanitizers [19]. In addition to these compounds, others able to act as NO-donors may play a crucial role in skin health [9]. The use of NO in cosmeceuticals is highly beneficial due to its multiple functions in regulating blood circulation, promoting healing, modulating the immune response, and protecting against damage caused by UV radiation. Additionally, NO has antimicrobial properties that help prevent skin infections [10,20]. Thus, integrating cyanobacterial aqueous extracts into skincare formulations may represent a promising advancement in creating innovative and eco-conscious formulations in the field of dermatology.

Given the multifaceted role of NO in skin health and its therapeutic potential, it is essential to explore novel sources that could enhance its availability and effectiveness. Cyanobacteria, particularly those from the Cape Verde archipelago, may offer a promising avenue for such research. These microorganisms are known for their diverse bioactive compounds, with antioxidant and anti-inflammatory activity [21]. By investigating the aqueous extracts of cyanobacteria from this unique region, we aim to evaluate their potential as effective NO donors and to advance the development of innovative ingredients in the fields of cosmetics and dermatology. This approach not only aligns with the growing interest in natural and sustainable materials but also leverages the unique properties of cyanobacteria to potentially improve skincare and

therapeutic outcomes through enhanced NO-mediated mechanisms.

2. Material and methods

2.1. Cyanobacteria strains and biomass production

Ten filamentous strains were selected for this study: *Salileptolyngbya* sp. LEGE 181184, *Salileptolyngbya* sp. LEGE 181201, *Baaleninema* sp. LEGE 181148, *Salileptolyngbya* sp. LEGE 181187, *Neolyngbya* sp. LEGE 181188, *Nodosilinea* sp. LEGE 181189, *Salileptolyngbya* sp. LEGE 181150, *Leptothoe* sp. LEGE 181156, *Nodosilineales* LEGE 181157 and *Salileptolyngbya* sp. LEGE 181158. These strains, isolated from the marine environments of Cape Verde and preserved in the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) of the Interdisciplinary Center for Marine and Environmental Research (CIIMAR), were previously taxonomically identified in a recent study developed by our research [21]. The primary objective of this study was to expand the knowledge on the biological potential of these strains, exploring them as sustainable resources for the production of natural ingredients acting as NO donors. A scale-up culture scheme was implemented with a maximum capacity of 4 L, for biomass production. The strains were grown in Z8 medium [22], enriched with $10 \mu\text{g L}^{-1}$ of vitamin B12, and supplemented with 25 g L^{-1} of synthetic sea salts (Tropic marine, Berlin, Germany). The cultures were maintained in the bioterium of aquatic organism of CIIMAR (BOGA) at a constant temperature of 25°C , with aeration, under a controlled light intensity of $10\text{--}30 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and a photoperiod of 14 h light:10 h dark. The Cyanobacteria biomass was harvested by filtration with a net mesh (pore size $30 \mu\text{m}$) and the salt of the culture medium was removed by washing with distilled water. The fresh biomass was frozen and, subsequently, freeze-dried and stored at -20°C until extracts preparation.

2.2. Extracts preparation

Aqueous extracts were prepared after the biomass pre-extraction with acetone [21] following a sequential extraction scheme that allowed the profitability of the biomass and the obtaining of extracts of different polarities and with different biological activities. Briefly, after acetone extracts preparation (recently explored by us), cell debris were left to dry in a fume hood. Then, 2 g of dry biomass were suspended in 80 mL of distilled water and extracted for 5 min in an ultrasonic bath (Fisherbrand® FB15053). The supernatant was collected by centrifugation at $5000 \times g$ for 5 min at 4°C (Thermo Scientific™ HERAUS Megafuge™ 16R), to remove cell debris. This extraction process was repeated, re-extracting the biomass two additional times. The supernatants from each extraction were pooled, frozen, and lyophilized (Lyo-Quest, Telstar, Barcelona, Spain) under reduced pressure (0.1 mbar with the condenser at -47°C). The dried aqueous extracts were stored at -20°C until further chemical and biological analyses.

2.3. Cell assays

2.3.1. Cell lines

To predict the safety profile of aqueous extracts of Cyanobacteria as natural ingredients for dermatological applications, an initial in vitro cytotoxicity assessment was performed using three cell lines representative of different skin layers: HaCaT human keratinocytes (ATCC), 3 T3/L1 mouse fibroblasts (ATCC) and human endothelial cells. hCMEC/D3 cells. Cells culture and maintenance methods adhered to protocols described in our previous studies [23].

2.3.2. Cytotoxicity assessment

The cytotoxicity of the extracts was assessed using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described in a previous study [23]. Aqueous extracts were resuspended in distilled water and diluted with DMEM Glutamax medium

(Dulbecco's Modified Eagle Medium DMEM GlutaMAX™—Gibco, Massachusetts, USA), supplemented with 10 % (v/v) fetal bovine serum (Biocrom, Berlin, Germany), 0.1 % Amphotericin B (GE Healthcare, Little Chafont, United Kingdom) and 1 % Pen-Strep (penicillin-streptomycin, 100 IU mL⁻¹ and 10 mg mL⁻¹, respectively) (Biocrom, Berlin, Germany), before cells exposure. Briefly, keratinocytes, fibroblasts and endothelial cells were seeded in 96 wells plates at a density of 2.5×10^4 cells mL⁻¹, 3.3×10^4 cells mL⁻¹ and 1.0×10^5 cells mL⁻¹, respectively. After 24 h of adhesion, cells were exposed for 24 and 48 h to fresh medium supplemented with 1 % of extract to final concentrations of 12.5 to 200 µg mL⁻¹. DMEM and DMSO at 20 % were used as solvent and positive (cells death) controls, respectively. After 24 and 48 h of incubation, 20 µL of 1 mg mL⁻¹ MTT (Sigma-Aldrich) were added to each well, and the cell plates were further incubated at 37 °C for 3 h. Post-incubation, the culture medium was removed by aspiration, and the resulting purple formazan salts were dissolved with 100 µL of DMSO. Absorbance was measured at 550 nm using a Synergy HT Multi-Detection Microplate Reader (Biotek, Bad Friedrichshall, Germany) with GEN5™ software. Each assay was performed in quadruplicate and averaged, with cytotoxicity expressed as a percentage of cells viability relative to the solvent control (100 % viability). To ensure reproducibility, each experiment was independently repeated three times.

2.4. Chemical profiling of cyanobacteria extracts

2.4.1. Total phenolic content (TPC)

The TPC of the cyanobacterial extracts was determined using the colorimetric assay of Folin-Ciocalteu, according to Barroso et al. [24]. The dry extracts were resuspended in water. Briefly, a volume of 25 µL of each extract (10 mg mL⁻¹) was thoroughly mixed with 25 µL of Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA), 200 µL of Na₂CO₃ solution (75 g L⁻¹) and 500 µL of deionized water. After the incubation period (60 min at room temperature), the absorbance of the colored product was measured at 725 nm, using a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall, Germany) operated by GEN5™ software. Standard calibration curve ($y = 2.245x + 0.0119$, $r^2 = 0.9997$) was obtained with seven concentrations of gallic acid (GA) (0.016 to 0.5 mg mL⁻¹). TPC in each extract was expressed in µg of gallic acid equivalents (GAE) per mg of dry extract. Three independent determinations were carried out in triplicate.

2.4.2. Phycobiliproteins (PBPs) and total chlorophylls (TChls)

The PBPs and TChls present in aqueous extracts were quantified spectrophotometrically. For PBPs quantification, aqueous extracts were resuspended in water to a final concentration of 0.5 mg mL⁻¹. PBPs were determined by measuring the absorbances at different wavelengths (562, 615 and 652 nm), in a cell with 1 cm of optical path. For TChls quantification, aqueous extracts were resuspended in acetone (90 %) to a final concentration of 0.5 mg mL⁻¹. The corresponding formulas were applied, as previously described [25] [26]:

$$\text{Phycocyanin (PC)} = \frac{A_{615\text{nm}} - 0.474 \times A_{652\text{nm}}}{5.34}$$

$$\text{Allophycocyanin (APC)} = \frac{A_{652\text{nm}} - 0.208 \times A_{615\text{nm}}}{5.09}$$

$$\text{Phycocerythrin (PE)} = \frac{A_{562\text{nm}} - 2.41 \times \text{PC} - 0.849 \times \text{APC}}{9.62}$$

$$\text{Total Chlorophylls (TChls)} = 21.3877 A_{630\text{nm}} + 10.3739 A_{647\text{nm}} + 5.3805 A_{664\text{nm}} + 5.5309 A_{691\text{nm}}$$

The experiment was carried out in triplicate, and the results were expressed in µg of the respective PBP or TChls, per mg of dry extract.

2.4.3. Total carbohydrates (TCarb) analysis

The carbohydrates present in the cyanobacteria extracts were analyzed based on the method proposed by Masuko and co-workers [27], a sensitive phenol-sulfuric acid method that has been used for measuring neutral sugars in oligosaccharides, proteoglycans, glycoproteins, and glycolipids. Briefly, 100 µL of aqueous extracts (resuspended in ethanol 80 %) were mixed with 500 µL of H₂SO₄ and 100 µL of phenol (20 %), and allowed to incubate during 30 min, at room temperature. The absorbance of the formed chromophore was measured at 490 nm, using a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall, Germany) operated by GEN5™ software. Standard calibration curve ($y = 0.4458x - 0.0051$, $r^2 = 0.9993$) was obtained with five concentrations of D-(+)-Glucose (Gluc) (0.0625 to 1 mg mL⁻¹). The experiment was carried out in duplicate, and the TCarb were expressed in µg of Gluc equivalents per mg of dry extract.

2.5. Biological activities

2.5.1. Cell-free assays

2.5.1.1. Superoxide anion radical (O₂^{•-}) scavenging. The superoxide anion radical (O₂^{•-}) scavenging ability of the extracts was determined following a previously described method, with some modifications [28]. Aqueous extracts were resuspended in water, and six serial dilutions, starting from 10 mg mL⁻¹, were prepared for each extract to evaluate their dose-dependent activity and determine IC values. All reagents, as well as extracts dilutions, were prepared in phosphate buffer (19 µM, pH 7.4). In a 96-wells plate, 50 µL of each extract dilution were mixed with 50 µL of 166 µM β-nicotinamide adenine dinucleotide reduced form (NADH) solution and 150 µL of 43 µM nitrotriazolium blue chloride (NBT). Subsequently, 50 µL of 2.7 µM phenazine methosulphate (PMS) was added to each well. The radical scavenging activity was monitored using a Synergy HT Multi-Detection Microplate Reader (Biotek, Bad Friedrichshall, Germany) operated by GEN5™ software. Measurements were taken kinetically at room temperature for 2 min at 562 nm. Three independent assays were performed in triplicate. GA was used as a positive control. Results were expressed as the percentage of free radical scavenging compared to the untreated control. IC values and corresponding dose-response curves were calculated using GraphPad Prism® software (version 10, for macOS), with results reported as mean ± SD (µg mL⁻¹) from at least three independent assays performed in duplicate.

2.5.1.2. •NO scavenging. The •NO scavenging activity of cyanobacterial extracts was assessed using the Griess reaction, following Barbosa et al. [29]. Briefly, serial dilutions of extracts (7.81 to 1000 µg mL⁻¹) were incubated with a SNP solution (serving as •NO donor) in KH₂PO₄ buffer (pH 7.4) for 60 min at room temperature under light exposure. After incubation, Griess reagent was added, and the absorbance was measured at 562 nm. Results were expressed as the percentage inhibition of •NO production relative to the control. Quercetin served as the positive control. IC values and dose-response curves were calculated using GraphPad Prism® software (version 10, for macOS). Three independent assays were conducted in duplicate.

2.5.1.3. Lipoxigenase (LOX) inhibition assay. To investigate the inhibitory effect of cyanobacterial extracts on LOX, a modified procedure based on Fernandes et al. [30], was followed. Serial dilutions of extracts, up to a maximum concentration of 500 µg mL⁻¹, were prepared. Each extract (20 µL) was pre-incubated in a reaction mixture containing 200 µL of phosphate buffer (pH 9.0) and 20 µL of LOX (100 U/20 µL) for 5 min at room temperature. After incubation, 20 µL of the substrate (linoleic acid, 4.18 mM in ethanol) was added to initiate the reaction. Absorbance was measured continuously at 234 nm for 3 min using a Synergy™ HT plate reader (Biotek Instruments, Winooski, USA).

operated by Gen5™ Software. LOX inhibition was determined by comparing the reaction rates in the presence of the extracts to those of the untreated control. Each assay was performed in duplicate and repeated at least three times independently. Quercetin was used as positive control.

2.5.2. Cell assays

The murine macrophage cell line RAW 264.7 was used to evaluate the effect of Cyanobacteria extracts on *NO production. Cytotoxicity was measured using the MTT assay as reported before.

2.5.2.1. Cell culture conditions and maintenance. The murine macrophages cell line RAW 264.7 was maintained in Dulbecco's Modified Eagle medium (DMEM), supplemented with GlutaMAX™-I, 10 % of inactivated FBS and 1 % Pen Strep, in a humidified atmosphere of 5 % CO₂, at 37 °C. Cells used for experiments were grown in ventilated flasks with medium renewal every two days.

2.5.2.2. Cytotoxicity evaluation. The MTT assay was performed after 24-h incubation with the extracts, according to recognized methodologies with slight modifications [31]. Cells were treated with 20 µL of 1 mg mL⁻¹ MTT solution (dissolved in PBS), and absorbance of the formed formazan salts was measured at 550 nm to determine cells viability. Results were expressed in percentage face to the untreated control (DMEM). Each test was performed in duplicate and repeated at least four times.

2.5.2.3. Evaluation of nitric oxide levels in culture medium. The effect of the extracts on *NO levels in the culture medium was evaluated using a RAW 264.7 cells density of 3.5×10^4 cells/well, following a previously established protocol [31]. Cells were treated with extracts serial dilutions (12.5 to 200 µg mL⁻¹) for 24 h. Nitrite (NO₂⁻) accumulation, indicating *NO production, was measured in the cells culture supernatant using the Griess reagent, by measuring the absorbance of the chromophore at 562 nm. The percentage of *NO reduction was calculated and compared to untreated controls (Basal cells metabolism) and to LPS-stimulated cells. The results were expressed as the % of NO₂⁻ relative to the untreated control and compared with the LPS-stimulated cells. Four independent assays were performed in triplicate.

2.5.2.4. Determination of L-citrulline levels. The levels of L-citrulline in the RAW 264.7 cells supernatant were measured according to a previously described protocol, with slight modifications [29]. RAW 264.7 cells were seeded into 48-wells plates at a density of 3.0×10^5 cells mL⁻¹ and allowed to adhere for 24 h at 37 °C in a 5 % CO₂ atmosphere. Cells were treated with non-cytotoxic concentrations of aqueous Cyanobacteria extracts (ranging from 12.5 to 200 µg mL⁻¹), LPS (1 µg mL⁻¹) or DMEM, and followed a 24-h incubation. Afterwards, the culture medium was removed, cells were washed with Hank's Balanced Salt Solution (HBSS), and subsequently treated with 300 µL of L-arginine (200 µM), prepared in HBSS. After 2-h incubation, L-citrulline levels in the cell's supernatant were measured. For quantification, 250 µL of supernatant were combined with diacetyl monoxime (79 mM), antipyrine E (47.8 mM), and H₂SO₄ (7.5 M), and incubated for 25 min at 96 °C. After cooling, absorbance was recorded at 405 nm. Controls included RAW 264.7 cells without extract (Basal metabolism), and LPS-stimulated cells. The impact of cyanobacteria extracts on L-citrulline levels was compared to the LPS-stimulated control. Three independent experiments were performed in duplicate.

2.5.2.5. Polymyxin B (PB) neutralizing effects on cyanobacteria extracts. In order to screen the presence of Cyanobacteria polysaccharides in the aqueous extracts, capable of stimulating NO production by RAW 264.7 cells, the effect of the extracts was assessed in the presence of polymyxin B (PB), following a previously established protocol [32]. Briefly, the

RAW 264.7 cells (3.5×10^4 cells/well) were seeded in 96-wells plates and incubated for 24 h at 37 °C in a humidified atmosphere with 5 % CO₂. Serial dilutions of extracts (12.5 to 200 µg mL⁻¹) were prepared and pre-treated with two concentrations of PB (25 and 50 µg mL⁻¹, in PBS) during 24-h at 37 °C. After incubation, the cells supernatant was removed, and the PB-treated extracts were added to the cells. Following 24 h of exposure, 75 µL of cells supernatant from each well were transferred to a new 96-wells plate. The effect of the PB-treated extracts on NO production by RAW 264.7 cells was measured using the Griess reagent as before. Controls included RAW 264.7 cells in DMEM (Basal metabolism) and LPS-stimulated, as well as cells treated with LPS-PB and PB alone. The effect of the PB-treated Cyanobacteria extracts on *NO levels was compared to the basal control. Four independent experiments were conducted in quadruplicate.

2.5.2.6. Cytotoxicity of PB. Cytotoxicity of PB was analyzed using the MTT assay, according to Lopes et al. 2020, with slight modifications [31]. Briefly, RAW264.7 cells were plated at a density of 3.5×10^4 cells/well in a 96-wells plate. After 24-h incubation, the cells were treated with two concentrations of PB (25 and 50 µg mL⁻¹), pre-mixed with extract serial dilutions for 24 h at 37 °C in 5 % CO₂. The cells were then incubated with MTT for 45 min at 37 °C. The culture medium was removed, the colored formazan salts dissolved in 100 µL of DMSO, and the absorbance measured at 550 nm using a Synergy HT Multi-Detection Microplate Reader (Biotek, Bad Friedrichshall, Germany) with GEN5™ software. Each assay was performed in quadruplicate and averaged, with cytotoxicity expressed as a percentage of cell viability relative to the solvent control (considered 100 % viable). To ensure reproducibility, each experiment was independently repeated four times.

2.6. Photomicrographs

Photomicrographs of strain *Salileptolyngbya* sp. LEGE 181184 were captured using an Olympus BX41 microscope, equipped with an Olympus DP72 camera, and processed with the cellSens Entry v.2.3 software. The slides were prepared with aliquots of the culture mixed in a 1:1 ratio with Alcian Blue formulation [33]. Furthermore, ChinaInk was used to evidence the exopolysaccharides (EPS) surrounding the cyanobacterial colonies.

2.7. PCR amplification for detection of toxin-producing genes

PCR was used to amplify toxin-synthesis-related genes encoding microcystin (mcyA), the amino transferase (AMT) of the microcystin and nodularin synthetases complexes (mcyE), saxitoxin (sxtA, sxtG, and sxtI), anatoxin (anaC), and cylindrospermopsin (cyrJ) by using specific primer sets and PCR programs, as described previously [34] (See table A1 in the supplemental information online).

2.8. Cyanotoxins profiling by LC-MS

Lyophilized cell crude extracts and GFC Filters: biomass (10 mg) or filters from environmental water were treated with adequate homogenizing volume, 10 up to 100 mL, of aqueous MeOH (70 % (v/v)). The cells are then lysed by sonication on ice at 60 Hz for 5 × 1 min (VibraCell 50-sonics & Material Inc. Danbury, CT, USA). The homogenate is centrifuged (4495 g; 4 °C) for 5 min to remove cell debris. The resulting clear supernatant is collected in a glass beaker and stored at -4 °C. The remaining pellet is re-extracted with new extraction solvent and the supernatants combined. All the process its repeated if detected unbroken cells. The process was repeated three times. The extracts were diluted in 1 mg mL⁻¹ and subsequently filtered through a 0.22 µm pore membrane.

A well-established standard operation protocol was followed to determine cyanotoxins presence in the extracts. Cyanobacteria 70 %

methanol extracts were injected in a Liquid Chromatograph Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA), coupled with a Mass Spectrometry LCQ Fleet™ Ion Trap Mass Spectrometer (Thermo Scientific, MA, USA), with LC-column ACE Excel C18 (50 × 2.1 mm I.D., 1.7 μm, Batch: V17-1253, Avantor® ACE®, VWR, PT. The analysis of cyanotoxins followed a method described previously [35]. Data processing was executed using Xcalibur™ version 2 software from Waters, USA.

Compounds were identified by comparing their retention times and mass spectra with those of authentic standards. Identification of cyanotoxins was achieved by comparing the mass spectra of the samples, relative to external standards. Calibration curves were constructed using five different standard concentrations, selected to represent the concentration range found in the samples. The calibration plots and corresponding r^2 values, as well as the limit of detection for the analyzed cyanotoxins are displayed in Table A2.

2.9. Zebrafish embryo acute toxicity test (zFET)

The zFET was selected to predict the environmental safety of the cyanobacteria extracts, as new natural ingredients with application in the fields of cosmeceuticals and dermatology. The OECD guideline 236 [36], with some modifications, was used to assess lethal and sub-lethal effects of exposure of wild type zebrafish embryos to aqueous extracts. Breeding, maintenance and embryo collection were conducted in our certified BOGA, CIIMAR, Porto, in accordance with the ethical guidelines of the European Union Council (Directive 2010/63/EU) and the Portuguese Ministry of Agriculture, Sea, Environment, and Spatial Planning (Decree-Law no. 113/2013, 7 August) for the protection of animals used for experimental and other scientific purposes. The exposure was carried out in E3 medium (pH 8.6), at 28 °C, and adapted for 96-well plates. Briefly, fertilized eggs within the first hour post fertilization (hpf) were exposed to a logarithmic range of *Salileptolyngbya* sp. LEGE 181184 aqueous extract concentrations, up to 1 mg mL⁻¹, until 96

hpf. This sample was selected due to its high capacity to stimulate *NO production in macrophages (Fig. 1), and its availability in sufficient quantity for experimentation. Ten embryos were exposed, per condition, and daily inspected under a microscope, with solution renewal after each examination. Lethal (coagulation of fertilized eggs, absence of heartbeat or somite formation, and failure of tail detachment from the yolk sac) and sub-lethal (hatching time, tail deformities, presence of heart or yolk sac edemas) were recorded. The assay was repeated three times.

2.10. Statistical analysis

Statistical analysis was performed using IBM SPSS STATISTICS software (version 29.0.1.1 for MacOS, IBM Corporation, New York, USA, 2021). Data were analyzed for normality and homogeneity of variances by Kolmogorov–Smirnov and Leven's tests, then submitted to a one-way ANOVA using a Tukey's HSD (honest significant difference) as a post-hoc test, or to an unpaired *t*-test. A Pearson correlation test was used to compare normalized expression data between the chemical profiles and biological activities of cyanobacteria extracts. Dose-response curves and both IC values for cells and zebrafish median lethal concentration (LC₅₀) estimates were obtained by nonlinear fitting to a four-parameter logistic equation through the least squares statistical method using Graphpad Prism® software (version 10.2.3 and 8.2.1 for MacOS, respectively).

3. Results and discussion

In this study, aqueous extracts were obtained through a sequential extraction method, which offered several advantages in terms of yield and variability of pigments with different biological properties. This method allowed the efficient targeted-extraction of pigments with different chemical and biological characteristics, effectively separating lipophilic and hydrophilic ones. Furthermore, it maximized the total

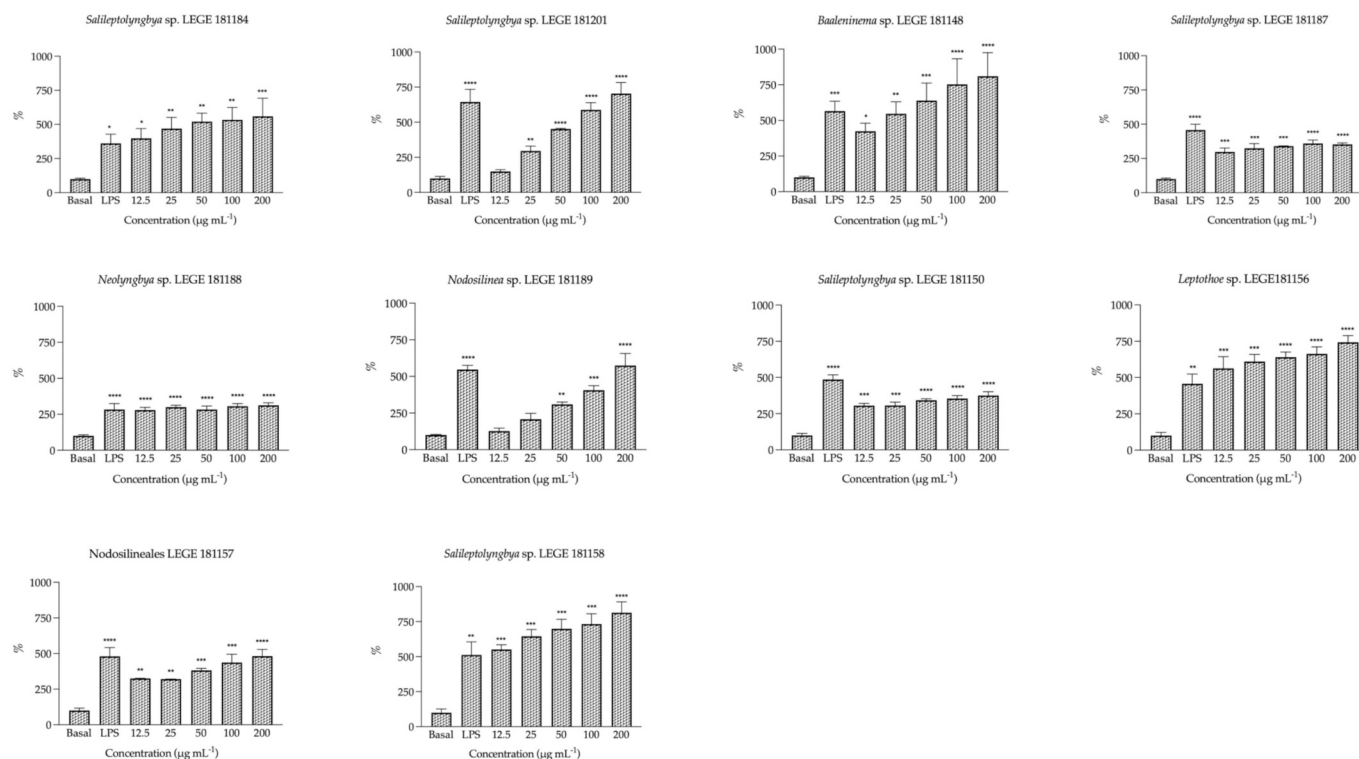


Fig. 1. Effect of cyanobacteria aqueous extracts on nitric oxide (*NO) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Results are expressed as percentage of nitrite (NO₂⁻), face to the untreated control (Basal metabolism) (mean ± SD three determinations, each performed in triplicate). **p* < 0.05; ***p* < 0.01; ****p* < 0.001, *****p* < 0.0001 (ANOVA, Turkey HSD multiple comparison test).

yield of the extraction, preserving the biological activity of the compounds from different classes by reducing interferences and making their characterization easier. The results showed that the yield of the extraction was significantly better for the aqueous extracts, as shown in Table 1. In all cases, the yield values of aqueous extracts were much higher than those obtained with acetone.

3.1. Cytotoxicity

The evaluation of cytotoxicity of natural extracts is crucial for the development of safe and effective ingredients, to ensure the absence of toxic compounds and prevent adverse effects. Furthermore, they promote the sustainable use of natural resources, reduce the need for animal testing and enable innovation through the discovery of new bioactive compounds. The use of keratinocytes (HaCaT), fibroblasts (3 T3/L1) and endothelial cells (hCMEC/D3) in cytotoxicity assays is essential to predict the safety and efficacy of natural extracts in cosmetics. Keratinocytes ensure that the barrier function of the epidermis is not compromised, fibroblasts assess the integrity of the dermis and healing, and endothelial cells ensure that there is no damage to microcirculation or induction of inflammation [37]. Altogether, these cell lines provide a holistic view of the effects of extracts, ensuring that these natural ingredients for dermatologic or cosmetic applications do not cause adverse effects before their commercialization.

In this study, *Neolyngbya* sp. LEGE 181188 was the only strain that showed cytotoxicity, in one of the cell lines analyzed, namely, 3 T3/L1, from the concentration 25 µg mL⁻¹, showing a cell viability of 60 %, after 48 h of exposure (*p* < 0.05). For all other strains, the aqueous extracts did not demonstrate cytotoxicity in the range of concentrations under study (*p* > 0.05) (Fig. A1). As a result, all extracts underwent additional analysis.

3.2. Phytochemical analysis

3.2.1. Total phenolic content (TPC)

The determination of TPC by the Folin-Ciocalteu method is widely used due to its simplicity, low-cost, speed and reproducibility, despite the limitations inherent to colorimetric assays, such as interference from other compounds. This method not only measures the TPC, but also allows inferring the antioxidant potential of the extracts, since phenolic compounds are recognized for their antioxidant properties [38]. As recently reported by us [21,28], acetone extracts often have a higher phenolic content than aqueous extracts, as many phenolic compounds are soluble in organic solvents, such as acetone. Furthermore, the sequential extraction with acetone followed by water, results in an initial removal of most phenolic compounds, leaving fewer phenolic compounds to be extracted in the subsequent aqueous phase. Although aqueous extracts sequentially obtained from acetone are poorer in total

Table 1
Yield (% w/w) obtained from sequential acetonic [28] and aqueous extractions.^a

Strains ^b	Solvents	
	Acetone	Water
<i>Salileptolyngbya</i> sp. LEGE 181184	1.22 ± 0.17	31.1 ± 3.60
<i>Salileptolyngbya</i> sp. LEGE 181201	0.55 ± 0.19	43.75 ± 5.30
<i>Baaleninema</i> sp. LEGE 181148	1.35 ± 0.08	28.5 ± 1.41
<i>Salileptolyngbya</i> sp. LEGE 181187	0.80 ± 0.16	25.25 ± 0.35
<i>Neolyngbya</i> sp. LEGE 181188	1.28 ± 0.04	26.13 ± 1.59
<i>Nodosilinea</i> sp. LEGE 181189	1.12 ± 0.24	27.00 ± 0.71
<i>Salileptolyngbya</i> sp. LEGE 181150	1.41 ± 0.39	39.0 ± 5.21
<i>Leptothoe</i> sp. LEGE 181156	1.91 ± 0.59	26.6 ± 5.26
<i>Nodosilineales</i> LEGE 181157	1.63 ± 0.56	31.72 ± 8.41
<i>Salileptolyngbya</i> sp. LEGE 181158	1.51 ± 0.32	23.35 ± 5.66

^a Values are expressed as mean ± SD of at least three extractions.

^b Taxonomic classifications.

phenols, they have advantages like the higher extraction yields, sustainability and safety profile. This approach can contribute to the development of innovative and environmentally responsible natural ingredients from cyanobacteria biomass.

The TPC values ranged from 14.49 up to 32.59 µg GAE mg⁻¹ dry extract, *Salileptolyngbya* sp. LEGE 181184 being the strain with the highest TPC value, followed by *Neolyngbya* sp. LEGE 181188 (*p* < 0.05) and *Nodosilinea* sp. LEGE 181189 (32.59, 26.97 and 26.42 µg GAE mg⁻¹ dry extract, respectively) (Table 2).

Although the TPC values of aqueous extracts are lower than that of acetonic extracts, the results become promising when converted into dry biomass, due to the excellent yield that aqueous extraction can provide. For example, *Salileptolyngbya* sp. LEGE 181184 and *Neolyngbya* sp. LEGE 181188 presented 10.95 and 6.74 mg GAE per gram of dry biomass, respectively. For the acetone extracts of these strains, the values were significantly lower, with 0.71 and 0.16 mg GAE per gram of dry biomass, respectively. In a previous work [23], using 70 % ethanol, *Cyanobium* sp. LEGE 07175 was the strain with the highest value in terms of dry biomass (1.09 mg GAE g⁻¹ dry biomass). In the study by Nabti et al. 2023 [39], the cyanobacterium used was *Limnospira platensis* (formerly *Arthrospira platensis*). The results obtained for total phenols in the methanolic extract were 28.33 mg GAE g⁻¹ dry biomass. Trabelsi et al., 2016 [40], showed a high production of phenolic compounds and flavonoids, especially under the influence of high temperatures in methanolic extracts by *Leptolyngbya* sp. (139 mg GAE g⁻¹ dry biomass) and the study concluded that optimizing cultivation conditions, mainly taking advantage of high temperatures, can significantly boost the production of these compounds in *Leptolyngbya* sp., thereby enhancing its antioxidant potential. Phenolic compounds are recognized for their positive effects on the skin, serving as antioxidants and anti-inflammatory agents [41].

Although the TPC values of aqueous extracts are lower than those of ethanolic and methanolic extractions previously reported, their advantage lies in sustainability and lower environmental impact, making them a preferable choice for ecological and sustainable practices. Furthermore, optimization of cultivation conditions, such as increasing temperature, can also be applied to aqueous extracts. This can enhance the production of phenolic compounds and other antioxidants, making aqueous extraction even more effective and beneficial. To our knowledge, there are no previous studies on TPC in aqueous extracts of marine cyanobacteria from Cape Verde archipelago, which opens new opportunities for research and development in this field.

3.2.2. Phycobiliproteins and chlorophylls

Present mainly in Cyanobacteria, PBPs are biodegradable, hydrophilic and non-toxic proteins, composed of phycobilins with tetrapyrrole structures. In Cyanobacteria, they are organized into phycobilisomes and play a role in photosynthesis, improving light capture and protecting their cells against photolysis under high light intensity. This protective role is crucial for the survival and efficiency of Cyanobacteria under varying light conditions [15]. PBPs are classified into three main categories: phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC), which have diverse biotechnological applications, being used as natural dyes, in biophotonics, in the food industry and with biomedical potential due to their fluorescent and antioxidant properties [15,38,42].

PBPs and TChls were quantified spectrophotometrically in aqueous extracts. Table 2 presents a detailed summary of the PBPs and TChls profile in the studied strains. *Salileptolyngbya* sp. LEGE 181150 and *Salileptolyngbya* sp. LEGE 181184 were the strains exhibiting the highest values for the PC pigment, showing no significant differences (222.76 and 220.75 µg mL⁻¹ dry extract, respectively). They were followed by *Baaleninema* sp. LEGE 181148, with a value of 211.60 µg mL⁻¹ dry extract (*p* < 0.05), correlating with the intense blue color present in their extracts. In contrast, *Nodosilineales* LEGE 181157 and *Leptothoe* sp. LEGE 181156, the strains presenting a vibrant purple-pink color in their extracts, exhibited a higher abundance of PE (275.04 and 159.05 µg mL⁻¹ dry extract,

Table 2
Total phenolic content (TPC), phycobiliproteins (PBPs), total chlorophylls (TChls) and total carbohydrates (TCarb) content in cyanobacterial aqueous extracts.^{a,b}

Strains ^c	TPC (μg (GAE) mg ⁻¹ DE ^d)	Phycobiliproteins (μg mg ⁻¹ DE)			Total chlorophylls (μg mg ⁻¹ DE)	Total carbohydrates (μg (CarbE) mg ⁻¹ DE)
		Phycocyanin	Allophycocyanin	Phycocerythrin		
<i>Salileptolyngbya</i> sp. LEGE 181184	32.59 ^A ± 0.38	220.75 ^A ± 4.18	35.59 ^C ± 1.84	14.43 ^F ± 0.40	2.53 ^B ± 0.16	167.71 ^{B,C} ± 6.34
<i>Salileptolyngbya</i> sp. LEGE 181201	16.24 ^C ± 0.88	17.70 ^I ± 0.28	6.41 ^E ± 0.44	67.34 ^C ± 0.18	0.73 ^{C,D} ± 0.18	76.81 ^D ± 12.36
<i>Baaleninema</i> sp. LEGE 181148	24.29 ^B ± 0.11	211.60 ^B ± 0.81	35.29 ^C ± 0.32	10.20 ^G ± 0.06	1.26 ^C ± 0.02	300.24 ^A ± 3.96
<i>Salileptolyngbya</i> sp. LEGE 181187	16.74 ^C ± 2.09	69.69 ^G ± 0.67	9.76 ^D ± 0.10	4.03 ^H ± 0.32	0.06 ^D ± 0.01	295.75 ^A ± 1.06
<i>Neolyngbya</i> sp. LEGE 181188	26.97 ^B ± 0.42	30.31 ^I ± 1.50	9.50 ^D ± 1.42	32.11 ^D ± 0.67	0.07 ^D ± 0.01	350.66 ^A ± 37.15
<i>Nodosilinea</i> sp. LEGE 181189	26.42 ^B ± 0.28	87.81 ^E ± 0.95	4.04 ^E ± 0.54	5.09 ^H ± 0.01	0.07 ^D ± 0.01	85.46 ^D ± 14.27
<i>Salileptolyngbya</i> sp. LEGE 181150	14.49 ^C ± 0.48	222.76 ^A ± 0.79	57.29 ^A ± 0.17	17.31 ^E ± 0.11	4.56 ^A ± 0.78	103.58 ^{C,D} ± 0.77
<i>Leptothoe</i> sp. LEGE 181156	14.93 ^C ± 0.27	76.10 ^F ± 0.13	37.05 ^C ± 0.28	159.05 ^B ± 0.14	0.45 ^{C,D} ± 0.04	196.89 ^B ± 24.51
Nodosilineales LEGE 181157	14.92 ^C ± 0.66	117.94 ^D ± 0.12	44.57 ^B ± 0.28	275.04 ^A ± 0.30	0.33 ^{C,D} ± 0.03	190.47 ^B ± 2.19
<i>Salileptolyngbya</i> sp. LEGE 181158	15.67 ^C ± 0.23	194.43 ^C ± 0.39	44.52 ^B ± 0.34	17.42 ^E ± 0.22	3.82 ^A ± 0.1	79.86 ^D ± 14.27

DE, dry extract; GAE, gallic acid equivalents; GlucE, glucose equivalents.
^a Mean ± SD of at least three independent experiments, performed in duplicate.
^b Different superscript letters in the same column correspond to statistical differences at $p < 0.05$ (ANOVA; Tukey's HSD).
^c Taxonomic classifications.

respectively) ($p < 0.05$). Regarding TChls, *Salileptolyngbya* sp. LEGE 181150 and *Salileptolyngbya* sp. LEGE 181158 were the strains which showed the highest values with no significant differences (4.56 and 3.82 μg mL⁻¹ extract, respectively), followed by *Salileptolyngbya* sp. LEGE 181184 (2.53 μg mL⁻¹ extract, $p < 0.05$).
In a previous study by Rodrigues et al. [43], it was shown that the strain *Leptolyngbya boryana* LEGE 15486 was one of the richest strains in PC, while *Leptothoe* sp. LEGE 11479 stood out for its high PE content (204.52 and 78.49 μg mg⁻¹ extract, respectively). The authors highlighted the importance of optimizing cultivation conditions to maximize PBPs production. High temperature, for example, may increase biomass and PC productivity. Furthermore, significant differences in the amount of PBPs between strains of the same species indicate that the production of these pigments is strictly individual and related to the characteristics of each cyanobacteria strain. In another work by Silva et al. [44], examining the pigment profile of extremophile cyanobacteria from the Volcanic Lake Chichonal, Mexico, it was shown the variability in the content of PBPs among different genera of cyanobacteria, such as *Tolypothrix* sp. and *Nostoc* sp. (50.61 and 57.66 μg mg⁻¹ extract, for PE and PC, respectively), emphasizing the importance of selecting specific strains to maximize the production of PBPs. In our work, the genera *Salileptolyngbya* sp. and *Baaleninema* sp. demonstrated superior PC production, while Nodosilineales and *Leptothoe* sp. stood out in the production of PE (Table 2), surpassing previous results. This diversity allows the selection of more efficient strains for specific PBPs production, thus reducing production time and associated costs, what translates into significant advantages in the field of biotechnology. For instance, a study on PC and PE extracts from *Limnospira platensis* (formerly *Arthrospira platensis*) and *Nostoc commune* [45] conducted cytotoxicity analyses and examined the release of inflammatory cytokines (IL-6 and TNF-α). The results demonstrated that PC and PE extracts did not induce significant inflammatory responses in human fibroblast cells, reinforcing their potential as safe anti-inflammatory agents for use in cosmetic and pharmaceutical products. Likewise, in another study [46], the aqueous extracts of *Planktothricoides raciborskii* and *Pseudochroococcus couteii* strains stood out for their ability to inhibit the production of the pro-inflammatory cytokine IL-1β (with inhibition of 57 % and 31 % at 50 μg mL⁻¹, respectively), suggesting that the presence of PC pigment, as well as other bioactive compounds, have great therapeutic potential

for use in medications or thermal therapies. Similarly, a study by Nowruzi & Hashemi [19], showed that PC and PE isolated from *Limnospira platensis* (formerly *Arthrospira platensis*) and *Nostoc*, were effective for skin care products such as soaps, anti-acne gels and hand sanitizers.
3.2.3. Total carbohydrates
Total carbohydrates were quantified spectrophotometrically in aqueous extracts. The method used for the quantification of total carbohydrates is widely recognized and commonly employed in scientific research, making it a reliable approach for measuring carbohydrate content in various samples, including cyanobacterial extracts [27].
According to Table 2, the total carbohydrates values ranged from 76.81 up to 350.66 μg GlucE mg⁻¹ dry extract. *Neolyngbya* sp. LEGE 181188, *Baaleninema* sp. LEGE 181148 and *Salileptolyngbya* sp. LEGE 181187 were the strains exhibiting the highest values for the total carbohydrates, showing no significant differences, (350.66, 300.24 and 295.75 μg GlucE mg⁻¹ dry extract, respectively). In comparison, the cyanobacterium *Nostoc muscorum* is reported in the literature [47] with a total carbohydrate content of 319.89 μg mg⁻¹ dry extract. This value is comparable to those obtained for *Neolyngbya* sp. LEGE 181188 (350.66 μg mg⁻¹), *Baaleninema* sp. LEGE 181148 (300.24 μg mg⁻¹), and *Salileptolyngbya* sp. LEGE 181187 (295.75 μg mg⁻¹). Such results highlight the significant potential of *Desmonostoc muscorum* (formerly *Nostoc muscorum*) for carbohydrate production, which is in the range of the highest-performing strains identified in this study. Furthermore, to the best of our knowledge, there is no evidence of other cyanobacterial strains exhibiting higher total carbohydrate values than those obtained herein. The data from the previously reviewed paper [48], as well as the most recent analysis, do not show any strain outperforming *Neolyngbya* sp. LEGE 181188, *Baaleninema* sp. LEGE 181148, and *Salileptolyngbya* sp. LEGE 181187. Although *Spirulina* species are often recognized for their high nutritional and biochemical potential, including carbohydrate production, the reviewed studies did not report carbohydrate values exceeding those observed in our strains. This further highlights the exceptional carbohydrate yield achieved in our study.
Carbohydrates play a vital role in Cyanobacteria, serving as primary energy reserves and essential structural components for their survival and adaptation to diverse environmental conditions. These compounds

enable cyanobacteria to withstand stressors such as nutrient scarcity, extreme temperatures, and high salinity, highlighting their ecological resilience. Typically found in the form of glycogen, sucrose, and extracellular polysaccharides (EPS), carbohydrates are present in the intracellular and extracellular matrices of cyanobacteria. They contribute to energy storage, structural integrity, and environmental interactions such as biofilm formation and nutrient capture [48]. On average, carbohydrates constitute approximately 20–30 % of the dry weight of cyanobacteria, depending on the species and environmental conditions, and can reach up to 70 % in *Limnospira maxima* (formerly *Spirulina maxima*) [49]. Quantification of total carbohydrates in cyanobacterial extracts therefore not only provides insights into their metabolic potential but also highlights their importance as a renewable resource for sustainable applications [48].

3.3. Biological activities

3.3.1. Antioxidant capacities

Oxidative stress is one of the main causes of tissues damage, being an ever-present condition in the majority of metabolic diseases and an important factor for the acceleration of skin aging. Free radicals, such as $O_2^{\bullet}$ and $\bullet NO$, are relevant physiological reactive species that can cause damage to skin cells and tissues. The antioxidant compounds present in cyanobacteria extracts can neutralize these free radicals, preventing premature aging and other organs damage [44].

In terms of $O_2^{\bullet}$ scavenging, *Salileptolyngbya* sp. LEGE 181184 was the most effective strain, with the lowest IC_{50} ($46.50 \mu g mL^{-1}$), followed by *Neolyngbya* sp. LEGE 181188 ($47.86 \mu g mL^{-1}$) and *Nodosilineales* LEGE 181157 ($63.24 \mu g mL^{-1}$), with no significant differences (Table 3). Furthermore, *Salileptolyngbya* sp. LEGE 181184 was one of the strains that also presented the highest values for TPC ($32.59 \mu g GAE mg_{dry}^{-1}$ extracts) and PC ($220.75 \mu g mg_{dry}^{-1}$ extract), while *Nodosilineales* LEGE 181157 presented the highest value for PE ($275.04 \mu g mg_{dry}^{-1}$ extract) (Table 2). It is worth highlighting that a significant negative correlation was found between $O_2^{\bullet}$ elimination and TPC (-0.440 , $p < 0.05$). Furthermore, a significant negative correlation between $O_2^{\bullet}$ elimination and total carbohydrates content (-0.592 , $p < 0.01$, for IC_{50}), PC and APC (-0.528 and -0.673 , $p < 0.05$, for IC_{25} respectively) and a negative, yet not significant, correlation between $O_2^{\bullet}$ elimination and

PE were found, suggesting the contribution of the phenolic compounds, carbohydrates and PBPs to radicals scavenging. Rodrigues et al. [43], also reported a negative correlation between $O_2^{\bullet}$ scavenging and PBPs content of different Cyanobacteria, corroborating our findings. In their study, *Leptolyngbya boryana* showed the best IC_{50} value for $O_2^{\bullet}$ scavenging ($49.24 \mu g mL^{-1}$). A recent work [50] has shown that micro-encapsulated C-PC from *Limnospira platensis* (formerly *Arthrospira platensis*) has high antioxidant capacity and is effective in neutralizing free radicals (IC_{50} ranged from 7.6 to $13.5 mg mL^{-1}$, for DPPH). Another study [51], demonstrated that PE from *Nostoc* had efficient scavenging effects ($IC_{50} = 57 \mu g mL^{-1}$ for $O_2^{\bullet}$). Sonani et al. [52], provided several protocols to facilitate the purification of PBPs. Although the isolation and purification of PBPs offers significant benefits in terms of purity, efficacy, consistency and potential for specific applications, the use of targeted aqueous extracts may be more advantageous for the development of natural ingredients, once they are less expensive, faster and easier to obtain. Moreover, the synergistic effects and multitarget activity of PBP-rich extracts brings an added value to cyanobacteria as sustainable bioresources for the production of natural ingredients with application in different dermatological affections.

Regarding $\bullet NO$ elimination, *Salileptolyngbya* LEGE 181150 was the only one capable of reaching IC_{50} ($964.58 \mu g mL^{-1}$). In terms of IC_{25} , *Salileptolyngbya* LEGE 101184 was the most effective, followed by the strains *Salileptolyngbya* LEGE 181150 and *Salileptolyngbya* LEGE 181158 (296.07 ; 301.13 and $334.95 \mu g mL^{-1}$, respectively) (Table 3). Silva and coworkers [44] showed that only the aqueous extract of the strain *Tolypothrix* sp. LEGE 221228 was able to reach IC_{50} ($1220 \mu g mL^{-1}$), and this strain also presented the best value for IC_{25} ($689.91 \mu g mL^{-1}$). In their work, the acetone extract of the strain *Tolypothrix* sp. LEGE 221228 had the best IC_{50} ($57.68 \mu g mL^{-1}$). Rodrigues et al. [43] revealed that aqueous extracts from *Cephalothrix lacustris* may be an exception, with an IC_{50} of $36.68 \mu g mL^{-1}$, since acetone extracts generally showed better values than aqueous ones. In our previous work [21] that characterized carotenoid extracts from the same strains presented herein, the IC_{50} results for $\bullet NO$ scavenging were better than for $O_2^{\bullet}$ and, in fact, statistical analyzes showed a significant negative correlation between zeaxanthin and chlorophyll and IC_{50} values ($p < 0.01$).

According to those previous studies, acetone extracts have been shown to be much more effective in terms of $\bullet NO$ scavenging, due to the

Table 3

Inhibitory concentration (IC) values ($\mu g mL^{-1}$) of cyanobacteria aqueous extracts. ^{a,b,c}

Strains ^d	$O_2^{\bullet}$ scavenging ($\mu g mL^{-1}$)		$\bullet NO$ scavenging ($\mu g mL^{-1}$)		LOX inhibition ($\mu g mL^{-1}$)	
	IC_{25}	IC_{50}	IC_{25}	IC_{50}	IC_{25}	IC_{50}
<i>Salileptolyngbya</i> sp. LEGE 181184	22.22 ^A	46.50 ^A	296.07 ^A	nd	52.48 ^{A,B}	430.76 ^E
	± 3.84	± 4.73	± 36.99		± 7.70	± 25.72
<i>Salileptolyngbya</i> sp. LEGE 181201	85.00 ^D	194.33 ^F	nd	nd	240.67 ^D	nd
	± 15.05	± 11.72			± 27.64	
<i>Baaleninema</i> sp. LEGE 181148	34.50 ^A	76.60 ^{A,B,C}	993.95 ^B	nd	79.51 ^{A,B,C}	376.41 ^{B,C,D}
	± 0.71	± 7.83	± 8.55		± 3.62	± 6.00
<i>Salileptolyngbya</i> sp. LEGE 181187	68.00 ^{B,C,D}	110.00 ^{C,D,E}	nd	nd	266.47 ^D	nd
	± 3.16	± 13.00			± 12.44	
<i>Neolyngbya</i> sp. LEGE 181188	24.50 ^A	47.86 ^A	nd	nd	170.42 ^{B,C,D}	nd
	± 5.51	± 1.57			± 35.13	
<i>Nodosilinea</i> sp. LEGE 181189	76.50 ^{C,D}	144.00 ^E	nd	nd	197.6 ^D	nd
	± 2.12	± 14.31			± 82.73	
<i>Salileptolyngbya</i> sp. LEGE 181150	24.29 ^A	112.18 ^{D,E}	301.13 ^A	964.58	28.49 ^A	254.62 ^A
	± 9.46	± 12.46	± 58.93	± 27.52	± 1.79	± 55.86
<i>Leptothoe</i> sp. LEGE 181156	41.10 ^{A,B}	nd	nd	nd	39.15 ^A	268.54 ^{A,B}
	± 5.24				± 18.21	± 15.72
<i>Nodosilineales</i> LEGE 181157	25.48 ^A	63.24 ^{A,B}	nd	nd	62.67 ^{A,B}	370.08 ^{B,C,D}
	± 6.81	± 24.67			± 9.88	± 30.78
<i>Salileptolyngbya</i> sp. LEGE 181158	51.22 ^{A,B,C}	404.59 ^B	334.95 ^A	nd	50.90 ^{A,B}	285.61 ^{A,B,C}
	± 24.20	± 14.30	± 71.37		± 11.38	± 52.00

^a Mean $\pm$ SD of at least three independent experiments, performed in duplicate.

^b nd: not determined.

^c Different superscript letters in the same column correspond to statistical differences at $p < 0.05$ (ANOVA; Tukey's HSD).

^d Taxonomic classifications.

presence of compounds such as carotenoids and chlorophylls, which are less polar and have a greater ability to eliminate this free radical. Despite this, there is a lack of studies for comparison on the scavenging of $\bullet\text{NO}$ by cyanobacteria aqueous extracts. Although the aqueous extracts were not efficient in eliminating $\bullet\text{NO}$, the IC_{50} values for $\text{O}_2^{\bullet-}$ scavenging were very interesting. These findings suggest that aqueous extracts of cyanobacteria could be effective in preventing oxidative stress-related skin aging by efficiently scavenging $\text{O}_2^{\bullet-}$.

3.3.2. Lipoyxygenase (LOX) inhibition

Inflammation is a critical physiological process necessary for healing and maintaining tissue homeostasis. However, when inflammation becomes chronic, it can contribute to the development of various diseases. The transition from acute to chronic inflammation involves intricate networks of cells and molecules, including enzymes, growth factors, cytokines, and biolipid mediators. Among these mediators are prostaglandins, leukotrienes, and thromboxanes, which are produced from arachidonic acid (AA) through the action of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes [53]. LOXs are a diverse group of enzymes that oxygenate polyunsaturated fatty acids, resulting in the formation of both pro-inflammatory and pro-resolving lipid mediators. These enzymes play a crucial role in various aspects of the inflammatory response, such as the recruitment of leukocytes (white blood cells), the regulation of vascular permeability, and the resolution of inflammation [43].

Targeting the LOX pathway, particularly in macrophages, offers a promising therapeutic strategy. By modulating this pathway, it may be possible to develop treatments that effectively reduce harmful inflammation while preserving the essential physiological functions of these enzymes. This approach aims to balance the need for resolving inflammation without disrupting the normal processes necessary for maintaining tissue health and homeostasis.

Salileptolyngbya sp. LEGE 181150 presented the lowest IC_{50} , followed by *Leptothoe* sp. LEGE 181156 and *Salileptolyngbya* sp. LEGE 181158 (254.62; 268.54 and 285.61 $\mu\text{g mL}^{-1}$, respectively). The extracts were tested up to a maximum concentration of 300 $\mu\text{g mL}^{-1}$. It is worth reporting that the values of IC_{25} were significantly lower, for the same strains (28.49; 39.15 and 50.90 $\mu\text{g mL}^{-1}$, respectively) (Table 3). Moreover, the IC_{25} values displayed by the extracts were in the same order of magnitude as that obtained with the positive control, quercetin ($\text{IC}_{25} = 31.77 \pm 7.61 \mu\text{g mL}^{-1}$), highlighting the potential of cyanobacteria extracts as natural ingredients for LOX inhibition. According to the statistical analysis, there is a negative correlation ($p < 0.01$) between PC, APC, and PE and TChls ($p < 0.05$) and the calculated IC values, suggesting the contribution of these PBPs and Chlorophylls to LOX inhibition. Rodrigues et al. [43] reported that aqueous extract from *Oscillatoria meretrix* (formerly *Leptolyngbya boryana*) LEGE 15486 and *Nodosilinea nodulosa* LEGE 06104, rich in PC, presented IC_{50} of 142.75 and 206.02 $\mu\text{g mL}^{-1}$, respectively, for LOX inhibition. In fact, the effect of purified PC on LOX has already been explored, showing the inhibitory properties of this PBP in the pro-inflammatory enzyme. For instance, Prasanth and co-workers reported that the PC-LOX interaction is of a function-freezing, protein-protein interaction in nature, and that the wide spectrum of properties of PC might be due to its function as a powerful protein hub showing non-specific protein-protein interactions [54]. Prabakaran and co-workers [55] also reported the ability of PC to inhibit LOX in 50 % for a concentration of 300 $\mu\text{g mL}^{-1}$, our results being more promising, with a significantly lower IC_{50} , highlighting the potential of the aqueous extracts for biotechnological application as LOX inhibitors.

Beyond PBPs, other compounds produced by cyanobacteria may contribute to LOX inhibition. In a work [56] investigating a new aqueous extract of *Limnospira platensis* (formerly *Arthrospira platensis*), enriched in PC and containing a high concentration of non-PC bioactive compounds, it was found that the inhibition of LOX was specifically associated with the non-PC fraction of the extract, suggesting that other

bioactive compounds may contribute to the activity observed. For instance, Fagundes et al. [57] provided evidence that phytosterols extracted from *Microcoleus autumnalis* (formerly *Phormidium autumnale*) are potentially responsible for the inhibition of LOX, among other neuroprotective activities. Furthermore, Morone et al. [21] revealed that acetone extracts from the same strains studied herein, exhibited IC_{50} values of the same order of magnitude, ranging from 202.43 to 367.26 $\mu\text{g mL}^{-1}$. In their study, the authors found a significant negative correlation between carotenoids and chlorophylls and IC values, suggesting that a variety of compounds produced by cyanobacteria contribute to the inhibition of LOX. Studies focusing the effect of Cyanobacteria extracts on the essential enzymes for arachidonic acid to eicosanoids conversion are scarce. Nevertheless, as for LOX, some reports point out the effect of Cyanobacteria-derived compounds in COX. Reddy and co-workers [58] revealed that PC from *Limnospira platensis* (formerly *Arthrospira platensis*) had potent ability to inhibit COX-2 activity at dosages between 1 and 30 $\mu\text{g mL}^{-1}$. Likewise, Leung et al. [59] reported that, in addition to promoting the downregulation of NF- κB , C-PC derived from *L. platensis* showed potent inhibition of COX-2. Pagels et al. [25], studying PBP-rich extract of *Cyanobium* sp., showed that their extracts were capable to reduce COX-1 and COX-2 enzymes activity, emphasizing the biotechnological importance of cyanobacteria targeted extracts as natural ingredients.

Thus, according to the literature, cyanobacterial extracts are rich in bioactive compounds that promote anti-inflammatory activity. Specifically, aqueous cyanobacterial extracts are particularly advantageous for use in cosmeceuticals as they are not only rich in these bioactive compounds but also sustainable, cost-effective, and environmentally friendly. These qualities make them ideal for natural and eco-conscious ingredients for both application in dermatologic and cosmeceutical formulations to treat skin affections with an associated inflammatory scenario.

3.3.3. Evaluation of NO levels in RAW 264.7 cells

The determination of NO levels in LPS-stimulated RAW 264.7 cells is widely used to evaluate the anti-inflammatory potential of different compounds, including cyanobacteria extracts. With this model, numerous inflammatory mediators can be initially measured to screen possible anti-inflammatory effects, sometimes being difficult to reach consensus on which mediators evaluate, and the rationality of measuring inflammatory mediators together with NO. In our study, we chose to do this approach by measuring the effect of the extracts on NO levels produced by RAW 264.7 cells. The results of NO levels produced RAW 264.7 cells in the presence of aqueous Cyanobacteria extracts are displayed in Fig. 1.

None of the extracts showed cytotoxicity to RAW 264.7 cells under the range of concentrations tested (Fig. A2). On the other hand, all extracts showed a significant capacity to increase the NO production by macrophages, after the 24-h incubation period, for all the concentrations investigated. This increment in NO production occurred in a dose-dependent manner, with the exception of *Salileptolyngbya* sp. LEGE 181187, *Neolyngbya* sp. LEGE 181188 and *Salileptolyngbya* sp. LEGE 181150 strains, where the levels of NO were similar for the tested range (Fig. 1). The *Nodosilinea* sp. LEGE 181189 extract showed a significant increase from the concentration of 50 $\mu\text{g mL}^{-1}$ and *Salileptolyngbya* sp. LEGE 181201 extracts from the concentration of 25 $\mu\text{g mL}^{-1}$. Four strains were found to exceed NO levels produced by RAW 264.7 upon LPS stimulation, namely *Salileptolyngbya* sp. LEGE 181184, *Baaleninema* sp. LEGE 181148, *Leptothoe* sp. LEGE 181156 and *Salileptolyngbya* sp. LEGE 181158, attesting the ability of these extracts to act as NO-producing ingredients. In a recent study carried out by us [21] using carotenoid-rich extracts obtained from the same strains, a contrary behavior was observed, with NO levels suffering a dose-dependent decrease. This highlights the importance of sequential and targeted extractions, that allow to obtain extracts with different biological activities from the same cyanobacteria strain. Rodrigues et al. [43] obtained

similar results for both aqueous and acetone extracts when studying other cyanobacteria strains.

Overall, data focusing cyanobacteria bioactivities points out the wide range of applications of their targeted extracts, which bioactivities widely vary depending on the strain and extraction methodologies. For instance, in a study conducted by Demay et al. [46], aqueous extracts of certain Cyanobacteria strains exhibited complex patterns of cytokine secretion, with some increasing the levels of one cytokine while decreasing another, complicating the assessment of their overall anti-inflammatory activity. The aqueous extracts of two strains, namely, *Planktothricoides raciborskii* PMC 877.14 and *Pseudochroococcus coutei* PMC 885.14, stood out for inhibiting one or more pro-inflammatory cytokines, such as IL-1 β and IL-6, while for the *Aliinostoc* sp. PMC 882.14 there was an increase in the secretion of IL-1 β and an inhibition of the secretion of IL-6. According to them, the anti-inflammatory properties of the aqueous extract could be conferred by the PC pigment and probably lipids. Nevertheless, for the strain *P. coutei* PMC 885.14, as it has a thick polysaccharide sheath and presents low levels of PC, the anti-inflammatory activity was attributed to the EPS as they are water-soluble. In a study by Zampieri et al. [60], EPS produced by *Phormidium* sp. ETS05 demonstrated anti-inflammatory potential in zebrafish models of inflammation, significantly reducing luciferase activity and the number of neutrophils in the inflamed area, without toxicity in human skin fibroblasts (HSF). These results suggested that *Phormidium* EPS may have important therapeutic properties due to their anti-inflammatory and pro-resolution activities. In contrast, the study by Swartzendruber et al. [61] investigated LPS from another cyanobacterium, *Geitlerinema* sp., and found that these LPS induce an inflammatory response in mice, increasing the production of pro-inflammatory cytokines such as IL-6, TNF- α , IFN- γ , and do not increase the production of anti-inflammatory cytokine IL-10. Furthermore, the study raised the possibility that microcystin, a toxin produced by many cyanobacteria, may also be responsible for the observed increase in NO, highlighting the importance of investigating the combined impact of LPS and microcystin on immune cells activation.

The divergence in results can be attributed to several reasons, including differences in the chemical composition of LPS and EPS between different species of cyanobacteria, the experimental environment (in vivo vs. in vitro), and the biological models used (zebrafish vs. mice).

Recognizing that PBPs constitute the majority of compounds present in the aqueous extracts prepared in this study and considering their anti-inflammatory activity previously recognized [62] and discussed herein, we hypothesize that LPS or cyanotoxins might be present in our extracts and stimulating macrophages to produce NO. To better understand the mechanisms underlying the increment in NO production, and the possible contribution of the different compounds in the cyanobacteria aqueous extracts, we started evaluating the effect in the NO-producing enzyme, iNOS, by measuring the levels of L-citrulline, an amino acid produced in stoichiometric amounts with NO. The colorimetric method employed herein takes advantage of the ureido group-specific reaction with diacetyl monoxime/antipyrine in the presence of sulfuric acid, using cell assay conditions that avoid the interferences promoted by the serum proteins and pH indicator present in cell culture medium [63]. With this assay, we intended to clarify if the increase in NO production observed comes from the overexpression of iNOS, promoted by compounds acting in a similar mechanism as that of bacterial LPS.

3.3.4. Determination of L-citrulline levels

L-citrulline is a non-essential amino acid that plays a crucial role in the urea cycle, where it helps eliminate ammonium from the body. In the context of inflammation and NO production, L-citrulline is of particular interest due to its direct relationship with the activity of the enzyme iNOS. During NO production, L-citrulline is generated as a byproduct of the conversion of L-arginine to NO by iNOS [9]. Therefore, measuring L-citrulline levels serves as a reliable indicator of iNOS expression, activity

and NO production. For this purpose, we selected the aqueous extract of *Salileptolyngbya* sp. LEGE 181184, the one available in higher amount, and presenting significant results in terms of NO production by RAW 264.7 cells in the presence of the extract. The results are displayed in Fig. 2.

A significant increase in the percentage of L-citrulline was observed compared to the basal levels, demonstrating that the extract increases iNOS expression, without being toxic to RAW 264.7 cells (Fig. A2). As iNOS overexpression occurs through TLR4 activation, we can presuppose that aqueous extracts may contain compounds that increase iNOS expression at mRNA levels, in a similar manner to bacterial LPS.

It is important to consider that Cyanobacteria, being Gram-negative, may contain LPS themselves, in their membranes, though, these LPS are generally less potent than those from pathogenic Gram-negative bacteria (LPS from *E. coli*) [64]. Moreover, Cyanobacteria may produce a variety of cyanotoxins, which are toxic compounds capable of causing adverse effects on both aquatic and terrestrial organisms. These cyanotoxins exhibit diverse activities, including hepatotoxic, neurotoxic and dermatotoxic effects. The presence of cyanotoxins in cyanobacteria extracts may also contribute to the observed NO overproduction, in addition to those caused by LPS.

To better understand whether the NO overproduction detected with our extracts is actually due to the presence of LPS or cyanotoxins, we screened out the presence of toxins in the extracts under study, as well as the presence of toxin-producing genes in the cyanobacteria biomass. As well, we performed an assay using polymyxin B (PB), an antibiotic that neutralizes LPS. This approach allows us to discern whether the stimulation of NO production in the presence of the extracts is predominantly caused by LPS or by other compounds, such as cyanotoxins.

3.3.5. Neutralizing effects with polymyxin B

PB is an antibiotic that specifically binds to LPS, neutralizing their ability to induce inflammatory responses. If our extracts contain LPS, they will bind to PB and, as expected, be reflected in a lower NO production by macrophages in the presence of PB-treated extracts. The aqueous extracts were pre-treated with PB for 24 h, RAW 264.7 cells were subsequently exposed to PB-treated extracts and, after 24 h of incubation, NO levels and cells viability were determined (Figs. 3 and A3).

The results showed a significant decrease in the NO produced by the cells, for all extract concentrations, when compared with the LPS-

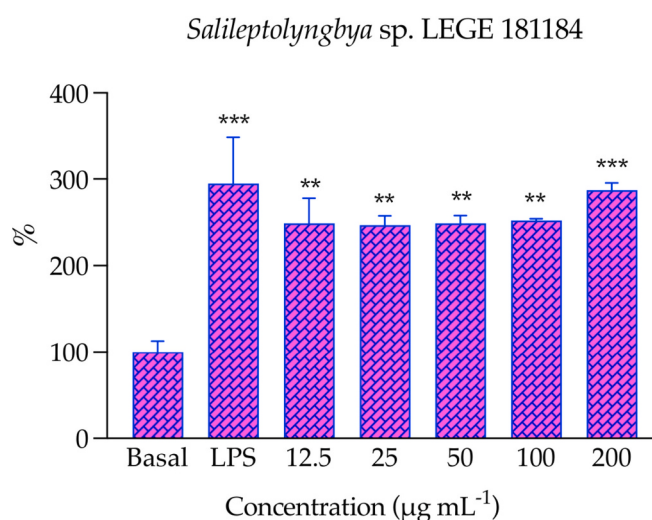


Fig. 2. L-citrulline production by RAW 264.7 cells in the presence of aqueous extract of *Salileptolyngbya* sp. LEGE 181184. Results are expressed as % of citrulline production relative to the untreated control (Basal, without LPS-stimulation). Results are expressed as the mean $\pm$ SD of at least four independent assays, performed in duplicate. ** p < 0.01, *** p < 0.001 (ANOVA, Tukey HSD).

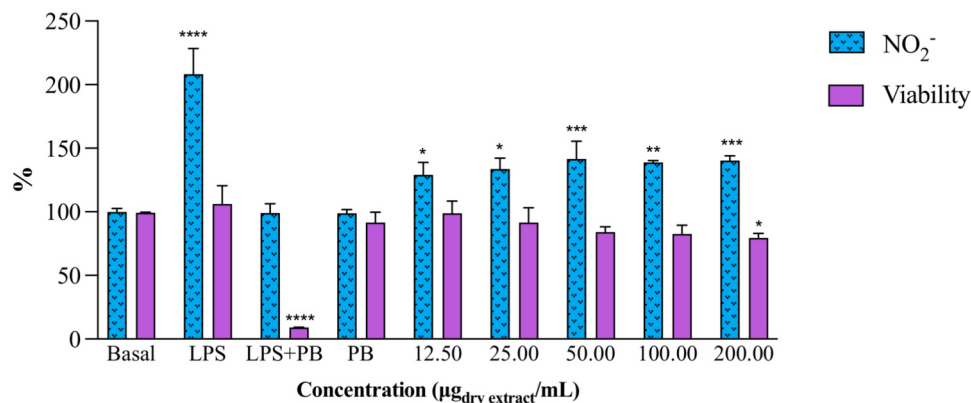
Salileptolyngbya sp. LEGE 181184

Fig. 3. Effect of *Salileptolyngbya* sp. LEGE 181184 aqueous extract pre-treated with Polymyxin B (PB, 25 µg mL⁻¹), on nitric oxide (*NO) produced by RAW 264.7 cells. Results are expressed as percentage of nitrite (NO₂⁻) face to the control (Basal) (mean ±SD of three determinations, each performed in triplicate). **p* < 0.05; ***p* < 0.01; ****p* < 0.001, *****p* < 0.0001 (ANOVA, Turkey HSD multiple comparison test).

stimulated cells, and as it was observed with the extract alone (as displayed in Fig. 1). Although the *NO production is still significantly superior to the basal levels (Fig. 3), it seems clear that PB-treatment has inactivated the compounds responsible for stimulating *NO production, indicating that the *NO overproduction previously observed (Fig. 1) may be attributed to the presence of LPS in the Cyanobacteria extracts (Fig. 3), corroborating the conclusions of the study by Swartzendruber et al. [61]. These findings provide additional evidence that LPS are primarily responsible for inducing the *NO overproduction in our assays.

It is worth mention that, for higher PB concentrations (50 µg mL⁻¹), a significant impact in cells viability is observed (Fig. A3), the same happening when PB is in combination with LPS, for both 25 and 50 µg mL⁻¹ (Fig. 3). Based on the observed results, it is evident that a concentration of 50 µg mL⁻¹ of PB cannot be used due to the occurrence of cell death. This finding emphasizes the importance of carefully selecting appropriate PB concentrations to avoid cytotoxic effects, ensuring that any observed changes in *NO production are solely due to the neutralization of LPS and not a consequence of compromised cell viability. Nevertheless, it has been well demonstrated that the LPS-neutralizing ability of PB lead to a NO production significantly lower than that previously observed with the extracts alone, indicating the presence of LPS-

like compounds in the cyanobacteria aqueous extracts.

As previously mentioned, carbohydrates can be found in the form of polysaccharides, in the intracellular and extracellular matrices of Cyanobacteria. To validate our assumptions about the presence of LPS-like compounds in Cyanobacteria, we examined the strain *Salileptolyngbya* sp. LEGE 181184 using optical microscopy, with the application of Alcian Blue and China Ink dyes. The Alcian blue formulation links to neutral LPS, allowing the detection of these compounds while the ChinaInk evidences the LPS surrounding the cyanobacterial colonies. As shown in Fig. 4, under the experimental conditions used in this study, *Salileptolyngbya* sp. LEGE 181184 is confirmed to be an LPS-producing strain, which aligns with the results depicted in Table 2 and with previously reported findings.

However, the possibility that other compounds, such as microcystins, may also contribute to this observation cannot be ruled out, highlighting the need to specifically identify and characterize the components of aqueous extracts responsible for these responses. In this regard, we have analyzed the Cyanobacteria strains under study for the presence of toxins.

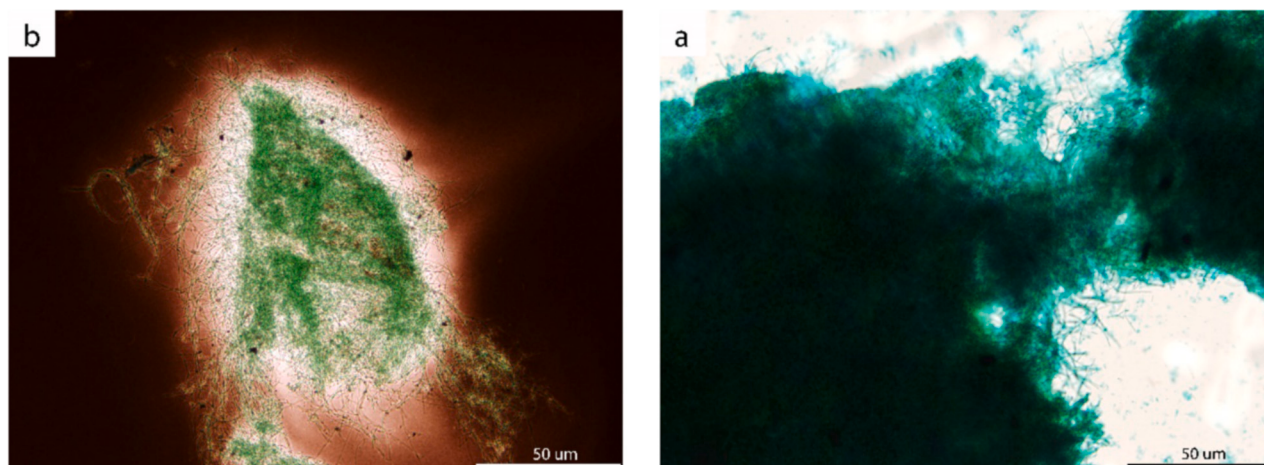


Fig. 4. Morphological analysis of *Salileptolyngbya* sp. LEGE 181184 with Alcian Blue staining (a) and with ChinaInk (b). All photos were taken with 10× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3.6. Screening of toxins production by PCR (Polymerase Chain Reaction) and by LC-MS

To complement our studies and investigate in more detail the potential contribution of compounds other than LPS to the increase in NO production, we performed a PCR assay to screen the presence of genes responsible for the production of cyanotoxins in Cyanobacteria extracts. The detection of specific genes, such as *mcyA* and *mcyE*, which are associated with the biosynthesis of microcystins and nodularins, respectively, allows us to identify strains of Cyanobacteria potentially producing these toxins [34]. This assay provides a more comprehensive understanding of the molecular composition of the extracts and helps clarify the role of different cyanotoxins in modulating the observed responses. The identification of such genes could also provide important clues about the mechanisms of toxicity and help direct future research towards mitigation and control strategies for toxic cyanobacteria.

For all the Cyanobacteria strains under study, PCR results were negative for the presence of the genes responsible for cyanotoxin production (Fig. A4). Additionally, and to complement this search, the LC-MS analysis of the extracts did not reveal any mass peak consistent with the presence of toxins (limits of detection in Table A2). This absence suggests that the increased NO production observed in our experiments, was predominantly mediated by cyanobacteria LPS, rather than by cyanotoxins such as microcystins or nodularins. Although the possibility of other unknown compounds contributing to NO overproduction cannot be completely ruled out, our data reinforce the hypothesis that cyanobacterial LPS are the main NO-production stimulating agents. These findings highlight the importance of focusing on the characterization of LPS and its interactions with the immune system, as well as on the identification of other possible bioactive molecules present in Cyanobacteria targeted extracts. Future studies could explore different strains and environmental conditions to broaden our understanding on the diversity and mechanisms of toxicity of Cyanobacteria.

3.3.7. Environmental safety of the NO-production stimulating extract

The Zebrafish Embryo Toxicity Test (ZFET) as outlined in OECD Test Guideline 236 [36], is a widely recognized, robust and reliable method used for both natural product discovery and environmental compatibility assessments of such products and/or extracts [65,66]. ZFET provides critical data on acute toxicity and animal development, serving as a key initial step in identifying exposure hazards in a biological system (in vivo). This is essential for evaluating potential environmental impacts of substances [67,68] such as natural extracts intended for various applications, including cosmetics [66,69,70].

In this study, ZFET was used as a proxy for hazard identification to depict major environmental problems, once release to the environment. For this study, as all aqueous extracts exhibited similar behavior in macrophages by stimulating *NO production, the extract from *Salileptolyngbya* sp. LEGE 181184 was selected due to its strong capacity for *NO stimulation, serving as a representative model for aqueous extracts. Additionally, this extract demonstrated no cytotoxicity in HaCaT, 3 T3/L1, and hCMEC/D3 cell lines (Fig. A1), and was available in sufficient quantity for experimental use. All three replicates of *Salileptolyngbya* sp. LEGE 181184 aqueous extracts met the required control criteria, with survival and normal development rates of at least 90 %. Nominal concentrations were used and the 96 h - LC₅₀ was determined to be 0.1712 mg mL⁻¹ with a 90 % confidence interval between 0.1542 and 0.1858 mg mL⁻¹ (Fig. 5). Sublethal effects, such as lordosis or edemas, were minimal. No effects on the hatching rate were observed.

These findings showed good agreement with the results obtained from the cellular cytotoxicity assay, in which, except for *Neolynghya* sp. LEGE 181188, no strains showed cellular cytotoxicity (Fig. A1).

While ZFET provides important initial data on the toxicity of natural extracts, inferring comprehensive environmental safety requires the integration of these data with additional tests and studies (e.g., multi-species testing, environmental fate, and bioaccumulation). This holistic approach ensures a more accurate and complete understanding of the

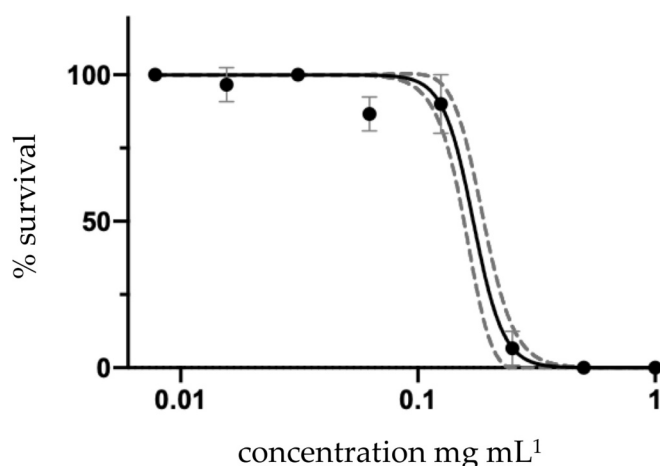


Fig. 5. Dose-response curve: *Danio rerio* (zebrafish) embryos survival at 96 h post fertilization (hpf) (full line) and respective confidence intervals (intermittent line) following exposure to *Salileptolyngbya* sp. LEGE 181184 aqueous extract.

potential ecological risks associated with the substance. Nevertheless, we compared our values with hazard classifications available in the literature. According to the OECD Harmonized Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures [71] our results fall above category III, in which the 96 h - LC₅₀ for fish is between >10 - ≤100 mg L⁻¹. This OECD document also refers to the possibility that compounds with 96 h - LC₅₀ > 100 mg L⁻¹ could be reconsidered in a new category, with less toxicity. In fact, this is in accordance with a hazard classification established by Passino and Smith [72] who characterized substances with acute toxicities between 100 and 1000 mg L⁻¹ as practically harmless.

Given these findings, our extracts can be considered environmentally safe, as the calculated IC values fall within concentration ranges that showed no cytotoxicity in the ZFET. For example, the IC₅₀ values for antioxidant activity against the O₂[•] radical ranged from 46.50 to 194.33 µg mL⁻¹, except for *Leptothoe* sp. LEGE 181156 and *Salileptolyngbya* sp. LEGE 181158 (Table 3). In the LOX inhibition assay, IC₂₅ values ranged from 28.49 to 197.64 µg mL⁻¹, except for *Salileptolyngbya* sp. LEGE 181201 and LEGE 181187 strains (Table 3).

3.3.7.1. Potential cosmetic and dermatologic applications of cyanobacteria extracts as NO donors. NO is a diffusible mediator recognized for its dualistic nature, the regulation of its levels being a key point for maintaining the organism's homeostasis, and for acting in specific situations of different nature. While inappropriate inducible NOS upregulation is implicated in a multitude of dermatologic disorders, there are specific situations where NO can represent a potent cosmeceutical with legitimate biological activity by conferring beautifying and protective effects. While it is true that much of the research directed at this mediator focuses on inhibiting its production, it is also true that in recent years its beneficial effects have been explored, particularly through substances that act as NO donors, both isolated and associated with nanoparticles. Special attention has been paid to the antimicrobial activity of NO, where the most significant aspect relies on the lack of resistance to date. Many resistant bacteria, such as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, among others, able to escape most of antibiotics, are susceptible to the effects of NO. In the field of cosmetics, NO donors are being explored as non-UV-based tanning agents capable of reducing photoaging and skin cancer risk [20].

Based on the results present herein, it seems evident the biotechnological interest of Cyanobacteria extracts as NO donors, with potential

application as broad-spectrum topical antimicrobial agents with activity against Gram-positive and -negative bacteria, fungi and parasites. Additionally, it is worth mentioning that Cyanobacteria-based formulations are already used in cosmetics, with *Limnospira* (formerly *Spirulina*) species being the most representative. This gives support to the safety of several strains of these Gram-negative microorganisms for humans use [73]. Beyond their low-cost, ease of production and high yields, the targeted extracts of the non-toxin producing Cyanobacteria from Cape Verde revealed a safe profile, with no cytotoxicity to the cell lines analyzed under the tested concentrations, as well as environmental safety demonstrated through the zFET acute toxicity test.

4. Conclusions

Aqueous extracts of the cyanobacterial strains *Salileptolyngbya* sp. LEGE 181184 and LEGE 181150, *Leptothoe* sp. LEGE 181156 and *Nodosilineales* LEGE 181157 have been shown to have substantial antioxidant and anti-inflammatory potential, evidenced by effective scavenging of the $O_2^{\bullet-}$ and inhibition of LOX, respectively. On the other hand, these non-toxin producing strains showed a significant stimulation of NO production in RAW 264.7 cells through the upregulation of iNOS expression, without presenting cytotoxicity to several cell lines studied.

These findings are particularly promising for the development of innovative natural antimicrobial ingredients acting against antibiotic-resistant strains, through a NO-donating mechanism. The antioxidant properties of these extracts, combined with the ability to promote vasodilation through increased NO synthesis, also make these strains valuable candidates for the development of innovative ingredients acting against cellulite and alopecia, as well as non-UV-based tanning agents. Although further studies are needed, namely exploring the stability of these extracts in topical release systems, their biotechnological advantages and environmental safety puts them at the forefront as serious candidates for the development of natural ingredients for dermatologic and cosmeceutical applications.

CRedit authorship contribution statement

Janaína Morone: Writing – original draft, Methodology, Investigation, Formal analysis. **Guilherme Scotta Hentschke:** Writing – review & editing, Methodology, Formal analysis. **Isabel Benta Oliveira:** Writing – review & editing, Methodology, Formal analysis. **Vitor Vasconcelos:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Rosário Martins:** Writing – review & editing, Supervision. **Graciliana Lopes:** Writing – review & editing, Validation, Supervision, Project administration, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2025.103952>.

Data availability

Data will be made available on request.

References

- [1] L. Litvinova, et al., Nitric oxide and mitochondria in metabolic syndrome, *Front. Physiol.* 6 (2015) 20.
- [2] S. Abdel Azim, C. Whiting, A.J. Friedman, Applications of nitric oxide-releasing nanomaterials in dermatology: skin infections and wound healing, *Nitric Oxide* 146 (2024) 10–18.
- [3] A.C. Barolet, I.V. Litvinov, D. Barolet, Light-induced nitric oxide release in the skin beyond UVA and blue light: red & near-infrared wavelengths, *Nitric Oxide* 117 (2021) 16–25.
- [4] C. Farah, L.Y.M. Michel, J.L. Balligand, Nitric oxide signalling in cardiovascular health and disease, *Nat. Rev. Cardiol.* 15 (5) (2018) 292–316.
- [5] M.R. Melis, A. Argiolas, Erectile function and sexual behavior: a review of the role of nitric oxide in the central nervous system, *Biomolecules* 11 (12) (2021).
- [6] Z. Bahadoran, et al., Vascular nitric oxide resistance in type 2 diabetes, *Cell Death Dis.* 14 (7) (2023) 410.
- [7] Cossenza, M., et al., Nitric oxide in the nervous system, in *Nitric Oxide*. 2014. p. 79–125.
- [8] M.M. Cals-Grierson, A.D. Ormerod, Nitric oxide function in the skin, *Nitric Oxide* 10 (4) (2004) 179–193.
- [9] A.B. Seabra, et al., Pharmacological applications of nitric oxide-releasing biomaterials in human skin, *Int. J. Pharm.* 630 (2023) 122465.
- [10] S. Settlemier, et al., Nitric oxide generating formulation as an innovative approach to topical skin care: an open-label pilot study, *Cosmetics* 8 (1) (2021).
- [11] M.C. Lombardo, et al., Nitric oxide functions as a positive regulator of root hair development, *Plant Signal. Behav.* 1 (1) (2006) 28–33.
- [12] M. Majewski, et al., Spirulina extract improves age-induced vascular dysfunction, *Pharm. Biol.* 60 (1) (2022) 627–637.
- [13] D.M. Villalpando, et al., Beneficial effects of Spirulina aqueous extract on vasodilator function of arteries from hypertensive rats, *Int J Vasc Med* 2020 (2020) 6657077.
- [14] F.M. Buratti, et al., Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation, *Arch. Toxicol.* 91 (3) (2017) 1049–1130.
- [15] L. Tounsi, et al., Microalgae: a promising source of bioactive phycobiliproteins, *Mar. Drugs* 21 (8) (2023).
- [16] J. Demay, et al., Natural products from Cyanobacteria: focus on beneficial activities, *Mar. Drugs* 17 (6) (2019).
- [17] A. Orona-Navar, et al., Alternative sources of natural pigments for dye-sensitized solar cells: algae, cyanobacteria, bacteria, archaea and fungi, *J. Biotechnol.* 332 (2021) 29–53.
- [18] S. Jha, et al., The radiant world of cyanobacterial phycobiliproteins: examining their structure, functions, and biomedical potentials, *Targets* 2 (1) (2024) 32–51.
- [19] B. Nowruzi, H. Hashemi, Impact of coated phycocyanin and phycoerythrin on antioxidant and antimicrobial activity of soap, anti-acne face wash and hand sanitizer gel, *Journal of Cosmetic Medicine* 8 (1) (2024) 18–33.
- [20] B.L. Adler, A.J. Friedman, Nitric oxide therapy for dermatologic disease, *Future Sci. OA* 1 (1) (2015) FSO37.
- [21] J. Morone, et al., Carotenoids from cyanobacteria modulate iNOS and inhibit the production of inflammatory mediators: promising agents for the treatment of inflammatory conditions, *Algal Res.* 83 (2024) 103729.
- [22] J. Kotai, Instructions for preparation of modified nutrient solution Z8 for algae, Norwegian Institute for Water Research 15 (1972) 155–164.
- [23] J. Morone, et al., Exploitation of filamentous and picoplanktonic Cyanobacteria for cosmetic applications: potential to improve skin structure and preserve dermal matrix components, *Mar. Drugs* 18 (9) (2020) 486.
- [24] M.F. Barroso, et al., Total antioxidant capacity of plant infusions: assessment using electrochemical DNA-based biosensor and spectrophotometric methods, *Food Control* 68 (2016) 153–161.
- [25] F. Pagels, et al., Bioactive potential of *Cyanobium* sp. pigment-rich extracts, *J. Appl. Phycol.* 32 (5) (2020) 3031–3040.
- [26] A. Bennett, L. Bogorad, Complementary chromatic adaptation in a filamentous blue-green alga, *J. Cell Biol.* 58 (2) (1973) 419–435.
- [27] T. Masuko, et al., Carbohydrate analysis by a phenol-sulfuric acid method in microplate format, *Anal. Biochem.* 339 (1) (2005) 69–72.
- [28] J. Morone, et al., Cosmetic application of Cyanobacteria extracts with a sustainable vision to skincare: role in the antioxidant and antiaging process, *Mar. Drugs* 20 (12) (2022) 761.

- [29] M. Barbosa, et al., Adding value to marine invaders by exploring the potential of *Sargassum muticum* (Yendo) Fensholt phlorotannin extract on targets underlying metabolic changes in diabetes, *Algal Res.* (2021) 59.
- [30] F. Fernandes, et al., Chemical profiling of edible seaweed (Ochrophyta) extracts and assessment of their in vitro effects on cell-free enzyme systems and on the viability of glutamate-injured SH-SY5Y cells, *Food Chem. Toxicol.* 116 (Pt B) (2018) 196–206.
- [31] G. Lopes, D. Clarinha, V. Vasconcelos, Carotenoids from Cyanobacteria: a biotechnological approach for the topical treatment of psoriasis, *Microorganisms* 8 (2) (2020) 302.
- [32] X.X. Lu, et al., Polymyxin B as an inhibitor of lipopolysaccharides contamination of herb crude polysaccharides in mononuclear cells, *Chin. J. Nat. Med.* 15 (7) (2017) 487–494.
- [33] J.D. Cruz, et al., Bioprospecting for industrially relevant exopolysaccharide-producing cyanobacteria under Portuguese simulated climate, *Sci. Rep.* 13 (1) (2023) 13561.
- [34] L. Benredjem, et al., First polyphasic study of Cheffia Reservoir (Algeria) cyanobacteria isolates reveals toxic picocyanobacteria genotype, *Microorganisms* 11 (11) (2023).
- [35] A. Galetovic, et al., Absence of cyanotoxins in Llayta, edible Nostocaceae colonies from the Andes Highlands, *Toxins (Basel)* 12 (6) (2020).
- [36] OECD, Test No. 236: Fish Embryo Acute Toxicity (FET) Test. 2013.
- [37] N.K. Karamanos, et al., A guide to the composition and functions of the extracellular matrix, *FEBS J.* 288 (24) (2021) 6850–6912.
- [38] J. Morone, et al., Cyanobacteria in cosmetics: a natural alternative for anti-aging ingredients, *Academic Press* (2022) 257–286.
- [39] B. Nabti, et al., Antioxidant and antimicrobial activities of *Spirulina* from the region of Tamanrasset, Algeria, *Journal of Herbal Medicine* (2023) 41.
- [40] L. Trabelsi, et al., Therapeutic properties in Tunisian hot springs: first evidence of phenolic compounds in the cyanobacterium *Leptolyngbya* sp. biomass, capsular polysaccharides and releasing polysaccharides, *BMC Complement. Altern. Med.* 16 (1) (2016) 515.
- [41] M.M. Rahman, et al., Role of phenolic compounds in human disease: current knowledge and future prospects, *Molecules* 27 (1) (2021).
- [42] V. Castro, R. Oliveira, A.C.P. Dias, Microalgae and cyanobacteria as sources of bioactive compounds for cosmetic applications: a systematic review, *Algal Res.* (2023) 76.
- [43] L. Rodrigues, et al., Anti-inflammatory activity of cyanobacteria pigment extracts: physiological free radical scavenging and modulation of iNOS and LOX activity, *Mar. Drugs* 22 (3) (2024).
- [44] R. Silva, et al., Pigments profile and antioxidant potential of extremophile cyanobacteria isolated from the Mexican Volcanic Lake Chichonal, *Algal Res.* (2024) 81.
- [45] B. Nowruzi, M. Zakerfirouzabad, Anti-inflammatory activities of phycoerythrin and phycocyanin on human fibroblast cells, *Phytomedicine Plus* 4 (3) (2024).
- [46] J. Demay, et al., Anti-inflammatory, antioxidant, and wound-healing properties of cyanobacteria from thermal mud of Balaruc-Les-Bains, France: a multi-approach study, *Biomolecules* 11 (1) (2020).
- [47] V.K. Patel, et al., Characterization of seven species of cyanobacteria for high-quality biomass production, *Arab. J. Sci. Eng.* 43 (1) (2017) 109–121.
- [48] L.S. Passos, et al., Content of lipids, fatty acids, carbohydrates, and proteins in continental cyanobacteria: a systematic analysis and database application, *Appl. Sci.* 13 (3162) (2023).
- [49] D.M. Arias, J. Garcia, E. Uggetti, Production of polymers by cyanobacteria grown in wastewater: current status, challenges and future perspectives, *New Biotechnol.* 55 (2020) 46–57.
- [50] W. Pan-Utai, S. Iamtham, Enhanced microencapsulation of C-phycocyanin from *Arthrospira* by freeze-drying with different wall materials, *Food Technol. Biotechnol.* 58 (4) (2020) 423–432.
- [51] B. Nowruzi, H. Fahimi, A.S. Lorenzi, Recovery of pure C-phycoerythrin from a limestone drought tolerant cyanobacterium *Nostoc* sp. and evaluation of its biological activity, *An. Biol.* 42 (2020) 115–128.
- [52] R.R. Sonani, et al., Recent advances in production, purification and applications of phycobiliproteins, *World J. Biol. Chem.* 7 (1) (2016) 100–109.
- [53] I.M. Rea, et al., Age and age-related diseases: role of inflammation triggers and cytokines, *Front. Immunol.* 9 (2018) 586.
- [54] S. Prasanth, et al., Phycocyanin of marine *Oscillatoria* sp. inhibits lipoxygenase by protein-protein interaction-induced change of active site entry apase: a model for non-specific biofunctions of phycocyanins, *Int. J. Biol. Macromol.* 165 (Pt A) (2020) 1111–1118.
- [55] G. Prabakaran, et al., Extraction and characterization of phycocyanin from *Spirulina platensis* and evaluation of its anticancer, antidiabetic and anti-inflammatory effect, *Int. J. Biol. Macromol.* 153 (2020) 256–263.
- [56] G.S. Jensen, et al., Antioxidant and anti-inflammatory properties of an aqueous cyanophyta extract derived from *Arthrospira platensis*: contribution to bioactivities by the non-phycocyanin aqueous fraction, *J. Med. Food* 18 (5) (2015) 535–541.
- [57] M.B. Fagundes, et al., Phytosterol-rich compressed fluids extracts from *Phormidium* autumnale cyanobacteria with neuroprotective potential, *Algal Res.* (2021) 55.
- [58] C.M. Reddy, et al., Selective inhibition of cyclooxygenase-2 by C-phycocyanin, a biliprotein from *Spirulina platensis*, *Biochem. Biophys. Res. Commun.* 277 (3) (2000) 599–603.
- [59] P.O. Leung, et al., Therapeutic effect of C-phycocyanin extracted from blue green algae in a rat model of acute lung injury induced by lipopolysaccharide, *Evid. Based Complement. Alternat. Med.* 2013 (2013) 916590.
- [60] R.M. Zampieri, et al., Anti-inflammatory activity of exopolysaccharides from *Phormidium* sp. ETS05, the most abundant cyanobacterium of the therapeutic Euganean thermal muds, using the zebrafish model, *Biomolecules* 10 (4) (2020) 582.
- [61] J.A. Swartzendruber, et al., Lipopolysaccharide from the cyanobacterium *Geitlerinema* sp. induces neutrophil infiltration and lung inflammation, *Toxins (Basel)* 14 (4) (2022).
- [62] M. Tabarza, V. Atabaki, T. Hosseinabadi, Anti-inflammatory activity of bioactive compounds from microalgae and cyanobacteria by focusing on the mechanisms of action, *Mol. Biol. Rep.* 47 (8) (2020) 6193–6205.
- [63] V. Costa, et al., Anti-inflammatory activity of olive oil polyphenols-the role of oleacein and its metabolites, *Biomedicines* 10 (11) (2022).
- [64] L. Pilotto, et al., Acute skin irritant effects of cyanobacteria (blue-green algae) in healthy volunteers, *Aust. N. Z. J. Public Health* 28 (3) (2004) 220–224.
- [65] C.D. Jayasinghe, U.A. Jayawardena, Toxicity assessment of herbal medicine using zebrafish embryos: a systematic review, *Evid. Based Complement. Alternat. Med.* 2019 (2019) 7272808.
- [66] C. Di Paolo, et al., The value of zebrafish as an integrative model in effect-directed analysis - a review, *Environ. Sci. Eur.* 27 (1) (2015).
- [67] I.B. Oliveira, et al., Toxicity of emerging antifouling biocides to non-target freshwater organisms from three trophic levels, *Aquat. Toxicol.* 191 (2017) 164–174.
- [68] C.A. Ferraz, et al., Chemical profile and eco-safety evaluation of essential oils and hydrolates from *Cistus ladanifer*, *Helichrysum italicum*, *Ocimum basilicum* and *Thymbra capitata*, *Ind. Crop. Prod.* 175 (2022).
- [69] Y. Ha, et al., Evaluation of cytotoxicity, genotoxicity, and zebrafish embryo toxicity of mixtures containing *Hyssopus officinalis*, *Morus alba*, *Engraulis japonicus*, and 27 other extracts for cosmetic safety assessment, *Mol. Cell. Toxicol.* 17 (2) (2021) 221–232.
- [70] M.A.P. Falcao, et al., Zebrafish as an alternative method for determining the embryo toxicity of plant products: a systematic review, *Environ. Sci. Pollut. Res. Int.* 25 (35) (2018) 35015–35026.
- [71] OECD, Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures. 2002: p. 249.
- [72] Passino, D.R.M.S., S. B., Acute bioassays and hazard evaluation of representative contaminants detected in great lakes fish. *Environ. Toxicol. Chem.*, 1987. 6: p. 901–907.
- [73] M.A. Furmaniak, et al., Edible cyanobacterial genus *Arthrospira*: actual state of the art in cultivation methods, genetics, and application in medicine, *Front. Microbiol.* 8 (2017) 2541.