Molecular Nutrition & Food Research

WILEY-VCH

Molecular Nutrition & Food Research

RESEARCH ARTICLE OPEN ACCESS

Assessment of Marine Microalgae's Bioactive Extracts Potential for Food Applications

V. Sousa[1] | M. Coelho[2] | J. Martins[1,3] | R. N. Pereira[1,3] | F. Maciel[1,3] | M. Pintado[2] | O. Dias[1,3] | A. A. Vicente[1,3] | P. Geada[1,3]

[1]Centre of Biological Engineering (CEB), University of Minho, Braga, Portugal | [2]CBQF—Centro De Biotecnologia e Química Fina—Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Porto, Portugal | [3]LABBELS Associate Laboratory, Braga/Guimarães, Portugal

Correspondence: V. Sousa (vitorsousa@ceb.uminho.pt)

Received: 12 May 2025 | **Revised:** 28 October 2025 | **Accepted:** 18 December 2025

Keywords: bioactive compounds | *Dunaliella salina* | microalgal bioactive extracts | nutritional bioactivity | *Pavlova gyrans*

ABSTRACT

The growing demand for sustainable and functional food ingredients has driven interest in marine microalgae as a source of bioactive compounds. This study evaluates the bioactivity, safety, and metabolite profile of extracts from *Dunaliella salina* and *Pavlova gyrans*, produced through two extraction methods: osmotic shock and bead milling with ethanol. Aqueous extracts were rich in peptides and amino acids, while ethanolic extracts contained higher pigment concentrations, underlining distinct nutritional biochemistry profiles. Both extracts exhibited significant antioxidant activity (4 mg mL^{-1}), with aqueous extracts demonstrating a stronger effect. Cellular antioxidant activity assays using the Caco-2 cell line confirmed comparable efficacy between extraction methods. Antibacterial tests revealed that *D. salina* extracts effectively inhibited *Listeria innocua*, *Escherichia coli*, and *Staphylococcus epidermidis*, while *P. gyrans* extracts showed activity against *L. innocua*, *E. coli*, *Bacillus cereus*, and *Pseudomonas fluorescens*. Additionally, both microalgae extracts demonstrated functional food potential by inhibiting α-glucosidase (27%–36%) and angiotensin-converting enzyme (21.7%–37.9%), suggesting antidiabetic and antihypertensive properties. Cell viability assays confirmed the extracts' safety for potential food applications. These findings support the sustainable use of *D. salina* and *P. gyrans* as innovative bioactive ingredients for functional and eco-friendly food formulations.

1 | Introduction

Marine microalgae have attracted increasing interest in recent decades, not only because of their important role in aquatic ecosystems but also because of their potential as a source of bioactive compounds with therapeutic and nutritional properties [1–5]. Among the various microalgae species, *Dunaliella salina* and *Pavlova gyrans* stand out for their unique characteristics. *D. salina* is widely known for its ability to accumulate high levels of carotenoids, especially β-carotene, in response to environmental stress conditions. This carotenoid has been associated with several bioactive activities, including antioxidant action and photoprotective potential [1, 4, 6]. Concomitantly, this microalga can produce high-quality protein [1]. On the other hand, *P. gyrans*, although less explored, is recognized for its richness in essential fatty acids, pigments, and proteins, which may contain promising bioactive peptides [2, 3]. The biochemical composition of microalgae is key to understanding their bioactive potential. Amino acids and peptides play essential therapeutic roles in maintaining cellular health, stimulating antioxidant, antimicrobial, antihypertensive, and immunomodulatory activities [7, 8].

The bioactivities of microalgae biomass have been widely investigated since it is a source of antioxidants (e.g., carotenoids, phenolics, and peptides) with protective functions on multiple

organs and tissues—heart, lungs, liver, kidneys, pancreas, eyes, and brain—against damage caused by reactive oxygen species (ROS), which is at the root of various diseases such as certain types of cancer, heart disease or atherosclerosis [7–11]. Presently, there is a pressing need to develop antibiotic alternatives due to the dramatic increase of multiresistant bacterial strains; thus, the search for new antibacterial compounds is now a priority [12]. Marine microalgae are emerging as a promising source of antimicrobial agents since they contain a variety of biocompounds that can inhibit microbes [10, 12–14]. Several studies have already demonstrated the potential of microalgae extracts against pathogenic microorganisms. For instance, the marine blue-green microalgae *Oscillatoria sp*. showed antibacterial capacity against gram-positive bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram-negative bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) with minimal inhibitory concentration (MIC) values of 30 and 25 μg mL^{-1}, respectively [13]. Another study showed that the marine microalgae *Chaetoceros gracilis* presented (MBICs) values of 16 and 32 mg L^{-1} for *C. albicans* and *C. parapsilosis*, respectively [12]. Another emerging research field is the bioactive peptides with antihypertensive potential, particularly those capable of inhibiting the angiotensin-converting enzyme (ACE), which could be relevant to the treatment and control of hypertension, a condition prevalent in the ageing population [7, 8]. Indeed, several studies have already demonstrated the potential of peptides from microalgae biomass such as *Spirulina* sp. [15], *Chlorella sorokiniana* [16], *Tetradesmus obliquus* [17], or *Chlorella vulgaris* [18] as a potential ACE-inhibitory agent. Despite the high bioactive potential demonstrated by the biocompounds extracted from microalgae biomass, it is necessary to evaluate the potential cytotoxicity of the extracts, which guarantees their safety and viability for future applications, whether in therapies, cosmetics, or food applications [10].

The biotechnological applications of compounds extracted from *D. salina* and *P. gyrans* are vast, covering industries ranging from cosmetics and pharmaceuticals to food and nutraceuticals [1, 2, 12]. In this regard, the bioactive properties of these microalgae make them promising sources for the development of new products for novel applications in future food systems [7, 10, 12, 14]. In addition, the production of microalgae has significant advantages, being a low-environmental impact and high productivity solution, in line with the principles of the circular economy and sustainability [5].

In this study, *D. salina*, a well-known microalga widely utilized in the food and nutraceutical industries, was selected as the reference species, whereas *P. gyrans*, a microalga with considerable but yet unexplored/uncharacterized biochemical potential, was chosen for comparative analysis. Aqueous and ethanolic extracts from both species were subject to a comprehensive evaluation of bioactivities—including antioxidant, antibacterial, antidiabetic, and antihypertensive properties—together with cytotoxicity assessments in human cell lines (Caco-2 and BJ5ta) and detailed biochemical characterization. The aim of this investigation was to deliver the first thorough evaluation of the bioactive potential of *P. gyrans* in direct comparison with an established reference species, thereby elucidating its potential as a novel source of functional ingredients for sustainable food and biotechnological applications.

2 | Material and Methods

2.1 | Microalgae Strains and Growing Conditions

The strains used in the present study were *Dunaliella salina* (RCC3579) and *Pavlova gyrans* (RCC1553) from the Roscoff Culture Collection, France (www.roscoff-culture-collection.org). These microalgae were cultivated autotrophically using an f/2-based medium for *D. salina* and Walne's medium for *P. gyrans* (see Supporting Information). The scale-up started in a 2 L flat bottom flask with a constant light supply of 100 μmolphotons·m^{-2}·s^{-1} and an air stream of 0.6 vvm, which was sequentially scaled up to a 10 L flat panel reactor under a constant light supply of 300 μmolphotons·m^{-2}·s^{-1}, a CO_2 stream of 6 mL min^{-1}, and an aeration rate of 1000 mL min^{-1}. After 7–11 days of growth, depending on the species, cultures reached the stationary phase. In the mid-stationary phase (2 days after stationary phase has been attained), cultures were harvested by centrifugation at 3000 g for 20 min (Centurion, model CR7000, 2/3 L, India) and the pellet was frozen (−20°C) for further lyophilization and then maintained at −20°C until extraction process.

2.2 | Microalgae Extracts Production

After harvesting the cultures, the biocompounds from microalgae biomass were extracted through two different methodologies: **i)** osmotic shock: 25 mL of deionized water was applied to 100 mg of freeze-dried biomass and this solution was stirred (250 rpm) overnight, and, **ii)** bead milling disruption: 32% (v/v) of glass beads (424–600 μm) and 10 mL of absolute ethanol were added to 100 mg of freeze-dried biomass followed by vortexing for 5 min. The supernatant was collected, and the procedure was repeated once again.

2.3 | Metabolite Profiling of the Extracts

2.3.1 | Identification and Quantification of Carotenoids

The pigment analysis was performed using an HPLC-DAD Agilent 1260 InfinityII system, following the method proposed by Wright et al. [19]. The analysis was conducted using a reverse-phase column C18 (2.1 × 150 mm, 2.7 μm), and the solvents employed at a flow rate of 1 mL min^{-1} included Solvent A—acetonitrile:water (90:10 v/v), Solvent B—ethyl acetate (100%), and Solvent C—methanol:ammonium acetate (80:20 v/v). Identification was based on retention times and UV–vis absorption spectra, compared with authentic standards (β-carotene, lutein, lycopene, β-cryptoxanthin, chlorophyll c, fucoxanthin, and diadinoxanthin). To prepare the samples for analysis, the extracts were collected and passed through a 0.22 μm filter from Millipore (Billerica, MA, U.S.), and the resulting solution was analyzed by HPLC.

2.3.2 | Peptide Size Profile

The molecular size distribution of the samples was analysed by High-Performance Size Exclusion Chromatography (HPSEC)

according to the procedure adapted from Coscueta et al. (2024) [20]. Analyses were carried out using an Agilent AdvanceBio SEC column (Agilent Technologies, London, UK) with 2.7 µm particle size, 130 Å pore size, and dimensions of 7.8 × 300 mm. The mobile phase consisted of 0.15 M sodium phosphate buffer (NaH2PO4) adjusted to pH 7, and separation was performed under isocratic conditions at a flow rate of 1 mL/min. Each sample (20 µL) was injected after filtration through 0.22 µm PTFE membranes. Chromatographic runs were performed on a Waters 2690 system equipped with a photodiode array detector (190–600 nm). Data acquisition and processing were carried out using Empower 3 software. The column was calibrated using a series of protein and peptide standards of known molecular weights: ovalbumin (44,300 Da), myoglobin (17,600 Da), cytochrome C (12,327 Da), aprotinin (6511 Da), neurotensin (1672 Da), angiotensin-II (1040 Da), the Tyr-Phe dipeptide (328.4 Da), and L-tryptophan (204 Da).

2.3.3 | Amino Acids

Amino acids were quantified in both hydrolyzed and non-hydrolyzed samples to distinguish total and free amino acid fractions. For total amino acids, samples (5 mg of dry biomass) were hydrolyzed in 6 N HCl at 110°C for 24 h under nitrogen. After hydrolysis, the pH was extremely acidic (pH < 1); therefore, 10 M NaOH was added to partially neutralize the solution to approximately pH 3.5, which is optimal for subsequent derivatization and HPLC analysis. This step prevents column damage and ensures consistent chromatographic performance. For free amino acids, extracts were directly filtered (0.22 µm) and analyzed without hydrolysis. Both fractions were derivatized using *o*-phthalaldehyde (OPA) and quantified by HPLC-DAD. The HPLC analysis was based on Agilent Poroshell HPH-C18 Column (2.1 × 200 mm, 5 µm, Agilent) as described by Fernandez Cunha et al. (2023) [21]. The analysis was performed using an Agilent 1200 series HPLC system consisting of an LC-20AB solvent delivery unit, a SIL-20A autosampler, a CTO-20A column oven (25°C), a G1321A FLD (excitation at 356 nm; emission at 4445 nm), and an LC Ver1.23 workstation (Agilent Technologies, Palo Alto, CA). Separation was completed using the Hypersil AA-ODS column fitted (Agilent Technologies, Palo Alto, CA). Two mobile phases were prepared: a) eluent A: 2.8 g of Na_2HPO_4, 7.6 g of $Na_2B_4O_7{\cdot}10H_2O$, and 0.064 g of NaN_3 (Sigma-Aldrich, Inc., St. Louis, USA) were dissolved in ultrapure water and the pH was adjusted to 8.2 with HCl, resulting in a total volume of 2 L; and b) eluent B: a mixture of 450 mL of methanol, 450 mL of acetonitrile (Fisher Chemical, USA), and 100 mL of ultrapure water was prepared to a total volume of 1 L. Several solutions and reagents were prepared as follows: c) Borate buffer (pH 9.5)—for 1 L, 6.2 g of H_3BO_3 were dissolved in ultrapure water, the pH was adjusted to 9.5 with 4 mol L^{-1} NaOH, and the solution was diluted to 1 L; d) Internal Standard (IS) Solution—homoserine (0.01 g) (Sigma-Aldrich, USA) was dissolved in 50 mL of 0.1 mol L^{-1} HCl to prepare a stock solution of 200 mg L^{-1}. This was then diluted tenfold with 0.1 mol L^{-1} HCl to obtain a 20 mg L^{-1} working solution; e) Reagent A—3 mL of the IS solution (20 mg L^{-1}) was mixed with 100 µL of mercaptoethanol (Fluka Analytical, USA) and 0.5 g of sodium tetraphenylborate (Merck, Germany); then, it was diluted to 25 mL with borate buffer. This solution was stored at 4°C and used within one week; f) Reagent B—0.6 g of iodoacetic acid (Sigma-Aldrich, USA) was dissolved in 15 mL of borate buffer, adjusted to pH 9.5 with 4 mol L^{-1} NaOH, and diluted to 20 mL with borate buffer; g) Reagent C—0.175 g of *o*-phthalaldehyde (OPA) (Sigma-Aldrich, USA) was dissolved in 5 mL of methanol, followed by the addition of 0.5 mL of mercaptoethanol. The solution was diluted to 25 mL with borate buffer, and nitrogen gas bubbled through it. The solution was stored at 4°C and covered with aluminum foil to protect from light.

A calibration curve was prepared using a 100 mg L^{-1} amino acid stock solution in 0.1 mol L^{-1} HCl. Standard solutions with concentrations ranging from 1 to 30 mg L^{-1} were prepared by diluting the stock solution accordingly in 0.1 mol L^{-1} HCl. Eluents were degassed using an ultrasonic bath. The fluorescence detection, with prior derivatization of the samples, was employed to quantify the free amino acids in the microalgae extracts. Results were expressed as mg of amino acid per g of dry biomass.

2.3.4 | Total Protein Content

The protein content was determined according to the Kjeldahl method. Briefly, a sample of approximately 0.5 g was digested in a mineralization block (Kjeltec Foss) with concentrated sulfuric acid (96% w/w) in the presence of a catalyst. The nitrogen content (N) was calculated based on the quantity of ammonia produced. The protein content was then determined using the following formula: %protein = %N × 6.25. A blank test was also conducted simultaneously. The assay was performed in triplicate.

2.3.5 | Total Phenolic Compounds

The total phenolic compounds were determined according to Loureiro et al. (2024) [10]. 0.2 mL of extract was mixed with 1.5 mL of Folin–Ciocalteu reagent (diluted 10:1 with distilled water) and rested on the stand in the dark, at room temperature, for 5 min. After this, 1.5 mL sodium bicarbonate solution (60 g L^{-1}) was added, and the mixture was incubated for 90 min, at room temperature, in the dark. The absorbance was measured at 750 nm. Gallic acid was used as the reference phenolic compound, and, regarding the calibration curve, a solution of gallic acid (150 mg L^{-1}) in 50% ethanol was prepared and diluted in the concentration range from 0 to 150 mg L^{-1}. The total phenolic content of the extracts was determined and expressed as gallic acid equivalents (GAE).

2.4 | Assessment of Bioactivities

2.4.1 | Antioxidant Activity

Antioxidant activity was measured by two assays—ABTS and DPPH—using ethanolic and aqueous extracts resulting from microalgae extraction, respectively, as previously described by Sousa et al. [22].

2.4.2 | ABTS

The ABTS radical scavenging assay was carried out by preparing a solution of 7 mmol L^{-1} of ABTS and 2.45 mmol L^{-1} of K_2S_2O

in distilled water. The reaction mixture was placed in the dark for 16 h at room temperature. Afterward, 250 µL of the solution was diluted in 22 mL of absolute ethanol. 50 µL of the sample was mixed with 1 mL of diluted ABTS solution. After 3 min of incubation at room temperature, the absorbance was measured at 734 nm. The ABTS radical scavenging activity was calculated considering Equation (1):

$$\text{Radical scavenging activity } (\%) = \left[\frac{(\text{Ac} - \text{As})}{\text{Ac}}\right] \times 100 \quad (1)$$

where A_s corresponds to the absorbance of the extract sample and A_c is the absorbance of the control sample.

2.4.3 | DPPH

The DPPH radical scavenging assay was carried out by preparing 0.1 mmol L^{-1} DPPH in an ethanol solution (50%). 150 µL of the previous solution was added to 50 µL of the sample, and after 30 min in the dark at room temperature, the absorbance was measured at 515 nm. The DPPH radical scavenging activity was analysed considering Equation (1).

2.4.4 | Antibacterial Activity

The crude extracts used for antibacterial activity analyses were obtained after lyophilization for the aqueous extracts, whereas the ethanolic extracts were dried under a N_2 stream and further resuspended in 1% dimethyl sulfoxide (DMSO). The Gram-positive and Gram-negative bacterial strains tested included *Escherichia coli* K12 EB2925, *Bacillus cereus* CECT 131, *Pseudomonas fluorescens* DSM 50090, *Staphylococcus epidermidis* CECT 231, and *Listeria innocua* CECT 4036, obtained from the stock culture of the Center of Biological Engineering, University of Minho, Portugal. The bacterial strains were cultured in Nutrient Broth, except *L. innocua*, which was grown in Brain Heart Infusion. The cultures were incubated for 24 h on a rotating shaker under 200 rpm at 30°C (*P. fluorescens* and *B. cereus*) and 37°C (*E.coli* K12, *S. epidermidis*, and *L. innocua*). The antimicrobial activity of the extracts was further tested using the minimum inhibitory concentration (MIC). The extract concentrations were sequentially diluted to 50, 40, 30, 20, and 10 mg extract mL^{-1} of 1% DMSO. A volume of 25 µL of the corresponding microorganism suspension (5×10^6 CFU mL^{-1}) in Mueller–Hinton was added to all plates. Negative controls (plates with 1% DMSO) and positive controls (plates without extract) were also included. MIC values were determined after overnight incubation and recorded as the lowest concentration of each test solution that inhibited the growth of all the microorganisms in the wells. The experiments were conducted in triplicate for each sample, and the average concentration of each triplicate was calculated.

2.4.5 | Cell Viability Assays

The crude extract was obtained from the aqueous extracts after lyophilization or from the ethanolic extracts after N_2 stream drying process. The crude extract was further resuspended in 1% (v/v) DMSO. The extracts' effect on Caco-2 and BJ5ta (ATCC, LGC Standards, Manchester, UK) cells' viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assay [10]. Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) obtained from Biowest (Nuaillé, France) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) obtained from Merck (Darmstadt, Germany), 1% (*v*/*v*) of penicillin/streptomycin (P/S) (Sigma-Aldrich (St. Louis, MO, USA). solution and 1% (*v*/*v*) of nonessential amino acids (NEAA) (Sigma-Aldrich (St. Louis, MO, USA). BJ5ta cells were grown in 4 parts of DMEM plus 1 part of M199 Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (*v*/*v*) of FBS, 1% (*v*/*v*) of P/S solution, and 10 µg mL^{-1} of hygromycin B (Sigma-Aldrich, St. Louis, MO, USA). Growing cultures were maintained at 37°C under a humidified atmosphere of 5% CO_2. Cells were seeded in 96-well tissue culture plates at 4×10^4 cells.well^{-1}, and cells were grown for 24 h to promote cell adhesion. Different extract concentrations diluted in DMEM culture medium (from 8 up to 1000 µg mL^{-1}) were added to cells. Cells only with DMEM was used as control (i.e., untreated cells). After 24 h of exposure, the medium containing the sample was removed, and the wells were washed with 200 µL of phosphate-buffered saline (PBS) (Lonza, Basel, Switzerland). Then, 100 µL MTT (0.5 mg mL^{-1}) was added to the 96-well plate, and it was incubated during 3 h at 37°C (5% CO_2 water-saturated atmosphere). Following the incubation period, the medium was removed, and 200 µL of DMSO was added to each well. Then, plates were placed on an orbital shaker for 30 min to dissolve entirely the formed formazan crystals. The absorbance was read at 570 nm using a SynergyTM HT Multi-mode Microplate Reader (Biotek Instruments, Winooski, VT, USA). The percentage of cell viability for all samples was assessed by Equation (2):

$$Cell\ viability\,(\%) = \frac{A\ sample}{A\ control} \times 100 \quad (2)$$

where A_{sample} is the absorbance value of the sample with cell culture, and A_{control} is the absorbance value of DMEM with cell culture.

2.4.6 | Antidiabetic Activity

The assessment of antidiabetic activity was performed by α-glucosidase inhibition, employing a colorimetric-based quantitative approach detailed by Oliveira et al. (2022) [23]. In this assay, 50 µL of microalgae extract of each tested condition was combined with 100 µL of 0.1 mol L^{-1} PBS (pH 6.9) containing α-glucosidase solution at a concentration of 1.0 U mL^{-1}. This mixture was incubated in 96-well plates at 25°C for 10 min. Following the preincubation, 50 µL of a 5 mmol L^{-1} p-nitrophenyl-α-D-glucopyranoside solution, prepared in 0.1 mol L^{-1} phosphate buffer (pH 6.9), was added to each well. The plate was then incubated at 25°C for 5 min, and absorbance measurements were taken at 405 nm using a microplate reader (Synergy H1, Vermont, USA). The results obtained were compared with a control, which contained 50 µL of a buffer solution instead of an active extract. Acarbose served as the positive standard at a concentration of 1 mg mL^{-1}. The inhibitory ability was calculated following

Equation (3) and expressed as percentage inhibition.

$$\alpha - \text{Glucosidase inhibition } (\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100 \quad (3)$$

where $A_{control}$ and A_{sample} correspond to the absorbance variation of the negative control and the sample, respectively.

2.4.7 | Antihypertensive Activity

The antihypertensive activity was determined through the ACE-inhibitory activity (iACE) using the fluorimetry assay described by Coelho et al. [24]. This method is based on the ability of the angiotensin-I converting enzyme to hydrolyse *o*-aminobenzoylglycyl-*p*-nitrophenylalanyl-proline (Abz–Gly–Phe(NO2)–Pro), generating the fluorescent product o-aminobenzoylgly-cine (Abz–Gly). The inhibitory activity was expressed as inhibited % of the maximum ACE activity. The Equation (4) was applied to calculate the percentage of ACE-inhibitory:

$$iACE\ inhibition\ (\%) = [(A_{CTL} - A_{BLK}) - (A_{SPL} - A_{SPLB})] \times \left[\frac{100}{(A_{CTL} - A_{BLK})}\right] \quad (4)$$

where A_{BLK}, A_{CTLl}, and A_{SPL} correspond to the absorbance of the blank, control, and the sample, respectively.

2.4.8 | Cellular Antioxidant Activity

The cell-based antioxidant activity (CAA) assay of the crude extracts was performed based on a method reported in the literature with some modifications [11]. Caco-2 cells were seeded at a density of 4×10^4/well on a 96-well microplate in 100 µL of supplemented DMEM. After a 48 h incubation period (at 37°C), the growth medium was removed, and the wells were washed with PBS. Cells were treated for 1 h at 37°C with 100 µL of the crude extract obtained from aqueous extracts or ethanolic extracts diluted in serum-free culture medium containing 25 µmol L^{-1} of 2',7'-dichlorofluorescin diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA). Then, 100 µL of 600 µmol L^{-1} 2,2′-azobis (2-methyl propionamidine) dihydrochloride (AAPH) (Sigma-Aldrich, St. Louis, MO, USA) was added to the cells. Then, the 96-well microplate was placed into a SynergyTM HT Multi-mode Microplate Reader (Biotek Instruments, Winooski, VT, USA) at 37°C and fluorescence intensity was recorded every 5 min for 1 h ($\lambda_{excitation}$ = 485 nm and ($\lambda_{emission}$ = 538 nm). The microplate included triplicate control wells (i.e., cells treated with DCFH-DA and AAPH) and blank wells (i.e., cells treated with DCFH-DA).

To calculate the CAA value of the samples, the area under the curve of fluorescence versus time was integrated with the following Equation (5):

$$\text{CAA unit} = 100 - \left(\frac{\int SA}{\int CA}\right) \times 100 \quad (5)$$

where $\int$SA is the integrated area under the sample fluorescence versus time curve, and $\int$CA is the integrated area from the control curve.

2.5 | Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 8 (GraphPad Software, La Jolla, USA). Two-way analysis of variance (ANOVA) with Tukey *post-hoc* test was applied to analyse all data. Data is represented as mean ± standard error of the mean (SD). Statistical significance was set for $p < 0.05$, and it is indicated by different superscript letters, according to their significance. Principal component analysis (PCA) was performed using OriginPro 2021 (version 9.8.0.200). The combination of these tools allowed us to highlight similarities and correlations between a set of preliminary results and extraction methods. Ellipses were established within each highlighted group with 95% of confidence degree.

3 | Results and Discussion

3.1 | Carotenoids and Chlorophylls Profiling

The results regarding the pigment content and profile of aqueous and ethanolic extracts from both microalgae—*P. gyrans* and *D. salina*—are shown in Figure 1. The extraction yields and total pigment contents obtained for microalgae under study are presented in Table S1. In both species, ethanolic extractions resulted in higher pigment recovery compared to aqueous extracts, reflecting the greater solubility of hydrophobic pigments in organic solvents. The extraction efficiency in *Pavlova gyrans* was 28.43% for the aqueous extract and 66.18% for the ethanolic extract, whereas *Dunaliella salina* exhibited slightly higher yields of 50.00% and 57.05% for the respective aqueous and ethanolic extracts. As expected, the pigment content in the raw biomass was higher than in the respective extracts, confirming that the extraction procedures recovered only a portion of the total pigment pool.

Notably, the *P. gyrans* ethanolic extracts resulted in high fucoxanthin, β-carotene, lutein, and β-cryptoxanthin levels. The concentration of fucoxanthin in the ethanolic extracts of *P. gyrans* was approximately 3.3 times higher than in the aqueous extracts, highlighting the potential of this carotenoid—known for its functional properties such as antioxidant, anticancer, anti-obesity, antidiabetic, and anti-photodamage activities, as well as its pharmacological effects including anti-inflammatory action, blood sugar regulation, and neuroprotection [25]. Similar trends were observed in the *D. salina* ethanolic extracts, where, for example, a higher lutein concentration than in the aqueous extracts was detected. Lutein also displays important biological functions related to the prevention of retinal degeneration and cardiovascular diseases, in addition to anti-cancer and antioxidant properties [5]. As expected, the significantly higher pigments' concentration observed in ethanolic extracts suggest that the pigments present in the microalgae biomass had a higher affinity for ethanol than for water, which results in improved extraction, as would be expected [26]. However, the diadinoxanthin was present in greater quantities in the aqueous extracts of both microalgae.

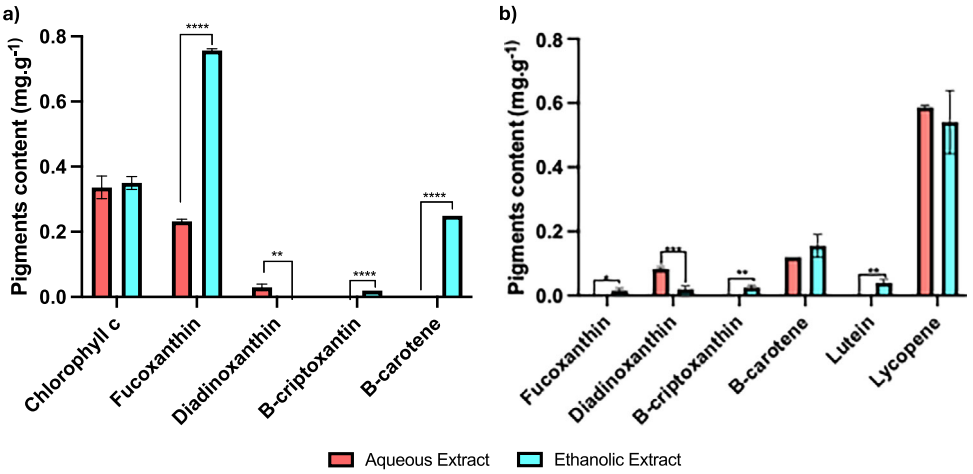

FIGURE 1 | Pigment content (mg g^{-1}) in aqueous (red) and ethanolic (blue) extracts from *Pavlova gyrans* (a) and *Dunaliella salina* (b). The graph presents the concentration of various pigments obtained after the extraction processes. Data are presented as mean ± standard error of the mean. Statistical significance was considered for (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, and (****) $p \leq 0.0001$.

3.2 | Protein Content

The total protein content of *D. salina* and *P. gyrans* was analysed. The results presented in Table S2 show that *P. gyrans* presented 1.7-fold higher protein content than *D. salina*. In addition, the extraction yields obtained for both species are summarized in Table S2. For *P. gyrans*, the aqueous extract exhibited a yield of 75.06%, whereas the ethanolic extract reached 8.74%. In *D. salina*, extraction efficiency was higher overall, with yields of 118% and 49.45% for aqueous and ethanolic extracts, respectively. These results indicate that the extraction efficiency varied substantially between solvents and species, with *D. salina* showing superior recovery in both extraction systems. This analysis was helpful to identify which of the biomasses could be a more promising source of protein compounds to be further investigated in this work.

3.3 | Peptide Profile

The peptide elution profiles (Figure 2) revealed distinct molecular weight distributions between *Dunaliella salina* and *Pavlova gyrans*. The molecular mass of the peptides associated with each elution peak was estimated by calibration of the SEC column with peptide and protein standards, providing an approximate correspondence between elution volume and molecular size, as previously reported for microalgal hydrolysates [27]. The chromatograms highlight distinct differences in protein size distribution across the microalgae species under study, with variations in absorbance peaks corresponding to different molecular weight ranges divided into six categories: >50, 10–50, 5–10, 3–5, 1–3, and <1 kDa. Each weight range revealed differences in peptide composition of the extracts.

P. gyrans and *D. salina* aqueous extracts showed a significant peak at the higher molecular weight range (>50 kDa), indicating a notable presence of large protein complexes (Figure 2b,e). In contrast, the ethanolic extracts of both microalgae exhibit lower absorbance values, suggesting less high molecular weight proteins. For the mid-range proteins (10–50 kDa), both microalgae aqueous extracts display prominent peaks, indicating that aqueous extracts contain a higher amount or variety of mid-sized proteins.

Both aqueous extracts from *P. gyrans* and *D. salina* showed significant absorbance peaks, in comparison to both ethanolic extracts, at low molecular weight ranges (5–10 kDa), suggesting the presence of small peptides or protein fragments; however, *P. gyrans* showed higher absorbance peaks, indicating a broader range of potential functional peptides. All the extracts studied exhibit distinct peaks in the lowest molecular weight ranges (1–3 kDa and <1 kDa), but the *P. gyrans* aqueous extract maintained higher absorbance values in these ranges, which may be associated with the presence of bioactive peptides, typically found in these lower molecular weight ranges [28].

The multiple absorbance peaks observed are consistent with a complex mixture of small peptides, whose contribution to biological activities remains unclear. *P. gyrans* demonstrated the highest absorbance across all molecular weight ranges, indicating a more abundant and diverse protein profile than *D. salina*. Moreover, *P. gyrans* aqueous extract showed the highest total protein content, reinforcing its potential as a rich source of peptides. The diversity in protein sizes, particularly the abundance of smaller peptides, indicates that *P. gyrans* contains not only a high protein quantity but also a wide variety of potentially bioactive peptides.

The molecular weight distribution (ratio, %) of protein-derived compounds from *D. salina* (a–c) and *P. gyrans* (d–f) is shown in Figure 2g. Distinct patterns were observed between species and extraction solvents. In both microalgae, aqueous extracts (b and e) were enriched in low-molecular-weight fractions, particularly <1 kDa (42.29% and 30.04%, respectively), whereas the ethanolic extracts (c and f) displayed a broader distribution with higher proportions in the 1–3 kDa range (48.28% and 40.03%, respectively). The total biomasses (a and d) were mainly composed of high-molecular-weight proteins (>50 kDa), consistent with their structural nature. Overall, aqueous extractions favored the recovery of small peptides and free amino acids, while ethanolic extractions yielded a more heterogeneous molecular profile. *D. salina* showed a higher proportion of low-molecular-

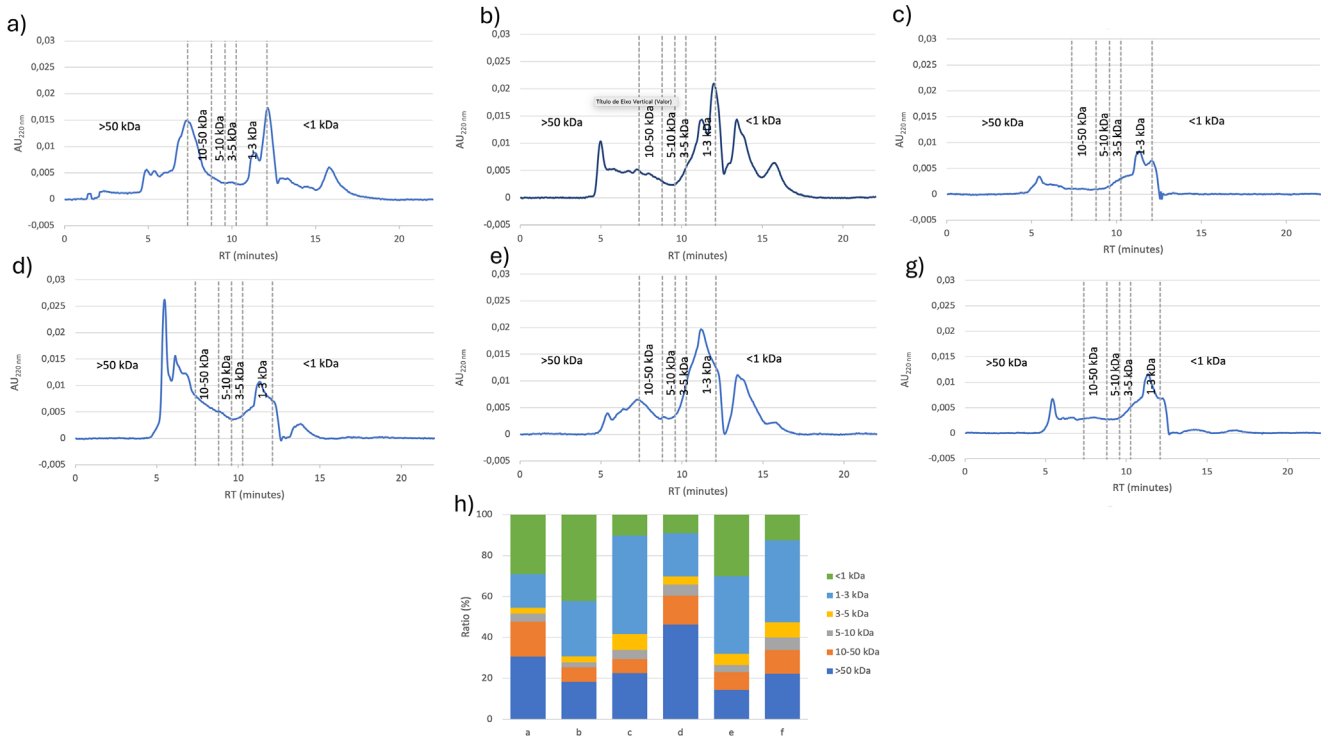

FIGURE 2 | Size-exclusion chromatograms of protein extracts from four microalgae extracts: (a) *Dunaliella salina* biomass, (b) *Dunaliella salina* aqueous extract, (c) *Dunaliella salina* ethanolic extract, (d) *Pavlova gyrans* biomass, (e) *Pavlova gyrans* aqueous extract, (f) *Pavlova gyrans* aqueous extract, and (g) molecular weight distribution (ratio, %) of protein-derived compounds from *Dunaliella salina* (a–c) and *Pavlova gyrans* (d–f). The *x*-axis represents the elution volume (mL), and the *y*-axis indicates absorbance at 280 nm (mAU). The chromatograms are divided into different molecular weight ranges (>50, 10–50, 5–10, 3–5, 1–3, and <1 kDa), showing the distribution of proteins of varying sizes in the extracts.

weight compounds compared to *P. gyrans*, which retained a larger fraction of macromolecules. Protease inhibitors were not included in the current extraction protocol, and some degradation may have occurred, which could have contributed to the observed abundance of low-molecular-weight peptides.

The combined analysis of Table S2 and Figure 2 highlights *P. gyrans*' superior potential as a source of diverse proteins and bioactive peptides, making it highly suitable for use in nutraceuticals, functional foods, pharmaceuticals, and other industries requiring a varied protein profile [29–31]. The presence of low molecular weight peptides across the studied extracts, especially in *P. gyrans* aqueous extract, indicates that these microalgae may also serve as a promising source of bioactive compounds with antioxidant, antihypertensive, or other properties [32] as further evaluated in this work.

3.4 | Amino Acids

The results regarding the amino acid content of aqueous and ethanolic extracts from *P. gyrans* and *D. salina* are shown in Figure 3. In *P. gyrans*, the aqueous extract exhibited a total amino acid concentration of 1.69 mg g^{-1}, while the ethanolic extract contained only 0.11 mg g^{-1}. Similarly, in *D. salina*, the total amino acid content reached 0.77 mg g^{-1} in the aqueous extract and 0.13 mg g^{-1} in the ethanolic extract. In summary, ethanolic extractions recovered only 6.52% and 17.23% of the amino acids extracted by water for *P. gyrans* and *D. salina*, respectively. A comparable trend was observed for essential amino acids (Figure 3b). For *P. gyrans*, the aqueous and ethanolic extracts contained 0.44 and 0.04 mg g^{-1}, respectively, corresponding to 9.32% recovery in ethanol relative to water. In *D. salina*, the aqueous extract yielded 0.36 mg g^{-1} of essential amino acids, while the ethanolic extract contained 0.05 mg g^{-1}, corresponding to 14.47% of the aqueous value. Overall, these results indicate that water was substantially more efficient than ethanol in recovering both total and essential amino acids. When comparing the two microalgal species, *P. gyrans* displayed higher total amino acid contents than *D. salina* in both extraction systems, suggesting interspecific differences in protein composition, as supported by the results previously observed in Section 3.2.

Aqueous extracts from *P. gyrans* exhibited consistently higher concentrations of several amino acids when compared to ethanolic extracts. Notably, the aqueous extracts resulted in elevated levels of aspartic acid, glutamic acid, alanine, and leucine. The concentration of aspartic acid in the aqueous extracts was approximately 39-fold higher than in the ethanolic extracts. Similar trends were observed for aspartic acid and glutamic acid, precursors of essential amino acids and vital components in protein metabolism [33], indicating a greater presence of amino acids in the aqueous extracts, consistent with their hydrophilic nature [34, 35]. Additionally, a significant increase of other amino acids such as valine, serine, and phenylalanine were recorded, further contributing to *Pavlova*'s overall amino acid richness in the aqueous extracts. The presence of high levels of alanine and leucine, both involved in energy metabolism and muscle

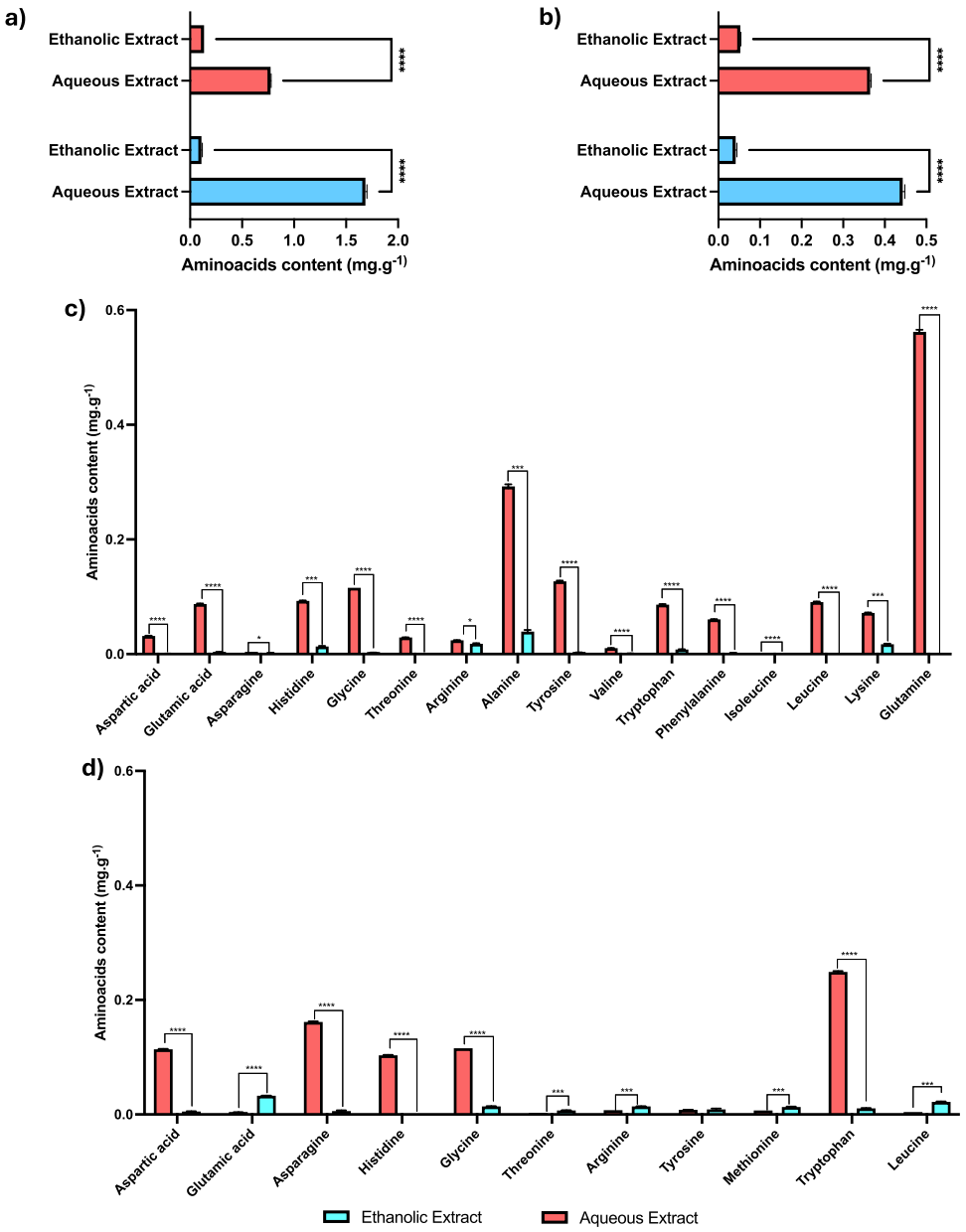

FIGURE 3 | Total amino acid content (mg·g^{-1}) in each extract (a), total essential amino acid content (mg·g^{-1}) in each extract (b), amino acid profile of *Pavlova gyrans* extracts (c), and amino acid profile of *Dunaliella salina* extracts (d) in the aqueous (red) and ethanolic (blue) extracts. Data is presented as mean ± standard error of the mean. Statistical significance was considered for (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, and (****) $p \leq 0.0001$.

protein synthesis [36, 37], further underscores *Pavlova*'s capacity for producing functional and potentially bioactive peptides. The *P. gyrans* extracts contain eight out of the nine essential amino acids, with only methionine not being detected. The broad range of essential and nonessential amino acids detected in *Pavlova* aligns with the findings of previous studies that highlighted its high protein content and diverse peptide profiles [3], as observed in the results from Figure 3a.

The amino acids measured in the *D. salina* extracts demonstrated the presence of five of the nine essential amino acids (Figure 3b). Also, the results showed inferior amino acid concentration across both extracts compared to the *P. gyrans* extracts. However, some amino acids were found in higher amounts in the aqueous extract of *D. salina* (such as tryptophan, asparagine, and histidine) than in the *P. gyrans* extracts. For instance, tryptophan, an essential amino acid precursor of serotonin, melatonin, and nicotinamide that regulates mood, sleep, and appetite [38, 39], was found in *D. salina* aqueous extract with a concentration *3-fold* higher than that in the same extract in *P. gyrans*. Additionally, in the *D. salina* extracts, contrary to what was observed in the *P. gyrans* extracts, methionine was found, which is an essential amino acid that plays important roles in mammalian metabolism associated with protein synthesis, DNA methylation, and the transsulfuration pathway [40]. In both aqueous and ethanolic extracts, *D. salina* displayed a relatively uniform and low profile of amino acids, with minimal variations between them. This could be due to the inherent metabolic pathways of *D. salina*. The amino acid profile and corresponding abundance in the biomass of *D. salina* suggest that this microalga might not be

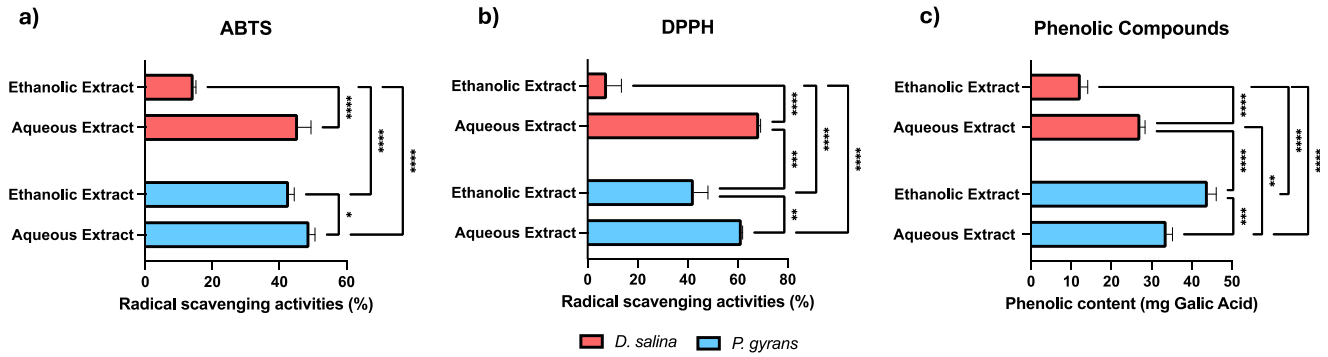

FIGURE 4 | Antioxidant activity by (a) ABTS (%) and (b) DPPH (%), and (c) the total phenolic (gallic acid equivalent mg L^{-1}) compounds of the aqueous and ethanolic extracts from *D. salina* and *P. gyrans*. Data is presented as mean ± standard error of the mean. Statistical significance was considered for (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, and (****) $p \leq 0.0001$.

suitable for applications requiring high amino acid or peptide content.

The comparative amino acid profiles of the microalgae under study provide valuable insights into their metabolic potential and protein synthesis capabilities. The superior amino acid profile of *P. gyrans*, especially in the aqueous extract, suggests its strong potential for use in nutraceuticals, functional foods, and pharmaceutical industries. The high concentrations of amino acids like glutamic acid and leucine are particularly beneficial for applications related to muscle growth and recovery, as well as general protein supplementation [36, 41]. Furthermore, the high presence of these amino acids, alongside smaller bioactive peptides, indicates that *P. gyrans* could serve as a valuable source of peptides with potential antioxidant, anti-inflammatory, or antimicrobial activities. In turn, *D. salina*, while not as rich in amino acids, still presents some value due to its unique metabolic characteristics. However, its lower protein and amino acid production suggested that it may be more suitable for niche applications where lower quantities of bioactive compounds are required or when other metabolic products, such as carotenoids, may be more relevant.

3.5 | Antioxidant Properties and Phenolic Content

The phenolic compounds are produced by microalgae in response to stressful conditions. These compounds can donate a hydrogen atom or an electron to free radicals, which makes them a potential antioxidant agent [10]. The total phenolic content of the extracts is presented in Figure 4. The extracts showed an amount of total phenolics that varied between 12.40 ± 1.69 and 44.00 ± 2.09 mg GAE. The highest amounts of phenolic compounds were detected in the *P. gyrans* extracts, namely in the aqueous extract. Phenolic compounds are associated with important bioactivities, such as antioxidant [10, 42] and antidiabetic [6, 23].

The radical scavenging activity was evaluated using the DPPH and ABTS assays to determine the antioxidant activity of the extracts. These methods simulate the presence of ROS through organic radicals that disappear in the presence of antioxidant compounds [43]. The DPPH and ABTS scavenging activity was determined for the aqueous and ethanolic extracts from both microalgae, at a concentration of 4 mg mL^{-1} (Figure 4). The lowest percentage of DPPH and ABTS scavenging activity was obtained for the *D. salina* ethanolic extract, with less than 15% for both methodologies. On the other hand, for both microalgae, the highest radical scavenging activity was observed in the aqueous extracts. It was also important to note that no statistically significant differences ($p > 0.05$) were observed between both aqueous extracts of the two microalgae. Some compounds, such as carotenoids, peptides, amino acids, or phenolic compounds, are well-known for their high antioxidant potential [1, 2, 7, 10]; the differences observed between aqueous and ethanolic extracts may, therefore, result from the presence and concentration of these compounds in the extracts.

Considering the extracts' composition, it was notorious that the highest antioxidant activity was detected in the extracts with the richest protein content in amino acids and peptides (see Sections 3.3 and 3.4). Indeed, some previous studies have already demonstrated the strong antioxidant activity of peptides [7, 8, 32]. Zhou et al. [32] demonstrated that peptides with low molecular weight (<1 kDa) had stronger antioxidant activity. Although carotenoids are well-known for their antioxidant potential [2, 44, 45], they seem to have a lower impact on this bioactivity in the present study. This might happen as the difference in the concentration of these compounds in both extracts was not significant, unlike what was found for amino acids, peptides, and phenolic compounds.

3.6 | Cellular Antioxidant Activity

The CAA assay has been widely used for studying and quantifying the antioxidant activity of phytochemicals, food extracts, dietary supplements, and microalgae extracts [11, 45, 46]. It has gained recognition as a biologically more relevant approach compared to conventional chemical antioxidant assays (e.g., ABTS, DPPH, FRAP, and ORAC), which solely measure chemical reactivity and fail to reflect the complexity of biological systems, as they do not account for factors such as bioavailability, stability, tissue distribution, or compound reactivity under physiological conditions [11, 47]. The utilization of cell cultures as models in the CAA assays provides an approach that is cost-effective, relatively fast, and addresses some issues of uptake, distribution, and metabolism [11, 46]. In the present study, the CAA of the aqueous and ethanolic extracts from both microalgae were measured with the Caco-2 cell

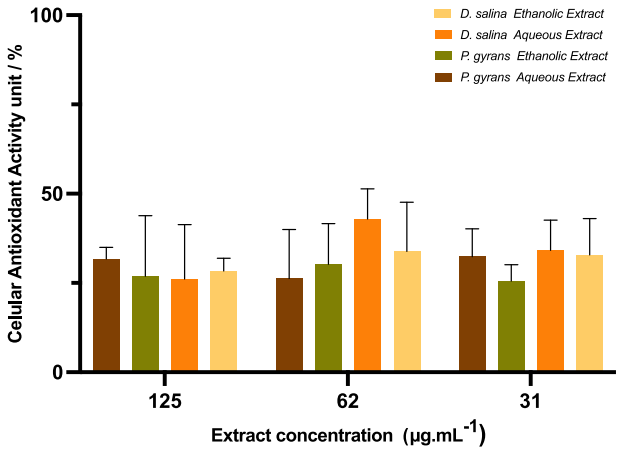

FIGURE 5 | Cellular antioxidant activity (CAA) of the aqueous and ethanolic extracts from *P. gyrans* and *D. salina* at concentrations of 125, 62, and 31 µg mL^{-1} in the Caco-2 cell line. Data is presented as mean ± standard error of the mean. Statistical significance was considered for (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$ and (****) $p \leq 0.0001$.

model at concentrations of 125, 62, and 31 µg mL^{-1} (Figure 5). Contrary to what was observed in the chemical antioxidant activity methods (Figure 4), when determining antioxidant activity using the CAA assay (Figure 5), there were no significant differences between the antioxidant quality of the four extracts under study ($p > 0.05$). The extracts showed an oxidative degeneration inhibition in the CAA assay between 25.5 and 42.9%. These results suggest that despite the greater antioxidant potential shown by some extracts in the chemical antioxidant activity assays, in biological models, the CAA assay showed that all of them have the same antioxidant potential for the Caco-2 cell line. Similar findings were reported by Wolfe & Rui [11], whose chemical methods assays not only yielded differences from their cellular antioxidant activity model (HepG2 cells), but also were different from each other.

3.7 | Antibacterial Activity

The results regarding the antibacterial activity of the aqueous and ethanolic extracts from both microalgae against five different types of bacteria (Gram-positive: *Bacillus cereus*, *Staphylococcus epidermidis*, and *Listeria innocua*; Gram-negative: *Escherichia coli* K12 and *Pseudomonas fluorescens*) are illustrated in Figure 6. The microalgae extracts under study were tested with five different concentrations:10, 20, 30, 40, and 50 mg mL^{-1}. Both aqueous and ethanolic extracts from *P. gyrans* and *D. salina* did not present a specific antibacterial activity for either Gram-positive or Gram-negative bacteria. *D. salina* ethanolic and aqueous extracts exhibited the same antibacterial potential. These extracts were effective against the bacteria *E. coli*, *S. epidermidis*, and *L. innocua* at concentrations of 10, 20, and 10 mg mL^{-1}, respectively. However, up to a concentration of 50 mg mL^{-1}, there was no inhibition for *B. cereus* and *P. fluorescens*, which may reflect the absence or insufficient concentration of compounds with antibacterial activity against these specific strains in the *D. salina* extracts. Regarding *P. gyrans* extracts, they showed similar antibacterial potential, although the ethanolic extract showed the same results at lower concentrations. The aqueous extract was effective against the bacteria *L. innocua, B. cereus*, and *S. epidermidis* with concentrations of 30, 50, and 10 mg mL^{-1}, respectively, while the ethanolic extract was effective against the same bacteria generally at lower concentrations: 20, 30, and 10 mg mL^{-1}. Additionally, *P. gyrans* ethanolic extract at a concentration of 50 mg $mL^{-1}$was also effective against *E. coli*. Many bioactive and pharmacologically active chemicals, particularly antibacterial compounds, are potentially produced by marine microalgae [12–14, 48, 49]. Based on the results obtained in the present study and considering the protein and pigment composition of each of the extracts, the antibacterial activity seen in the aqueous extracts was likely associated with the high presence of peptides and amino acids, while in the ethanolic extracts, the antibacterial activity might derive from a greater presence of pigments. The high antibacterial activity verified on *P. gyrans* ethanolic extract may be related to the higher content of fucoxanthin (3.3-fold) and the potential presence of polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) (although not quantified in the present study, *P. gyrans* is a well-known source of this polyunsaturated fatty acid [2, 3]), which would not be present in the aqueous extract due to its polarity. Both PUFAs and fucoxanthin have already demonstrated their antibacterial potential in other studies [12, 14, 48, 49]. Desbois et al. [50], for instance, revealed that EPA is active against a range of both Gram-positive and Gram-negative bacteria, including multi-resistant *S. aureus*, at micromolar concentrations. In another study, Saha et al. [51] demonstrated that the carotenoid fucoxanthin found on the surface of the macroalga *Fucus vesiculosus* acted as an inhibitor of bacterial settlement. Previous findings suggest that molecules with antibacterial action in microalgae are generally hydrophobic [12–14]. However, in the present study, we found that the aqueous extracts produced had a similar antibacterial potential to the ethanolic extracts, showing that the protein fractions of the microalgae under study have significant potential for antibacterial activity.

3.8 | Antihypertensive and Antidiabetic Activities

ACE is one of the leading regulators of blood pressure, with this enzyme being the main therapeutic target for the development of antihypertensive drugs through its suppression. The inhibition of ACE leads to a decrease in vasoconstrictor angiotensin II and an increase in vasodilator bradykinin, thus resulting in an antihypertensive effect [7, 24]. Several studies in the literature highlight the potential of microalgae's protein fraction, namely peptides, against hypertension [15–18]. In the present study, all the aqueous and ethanolic extracts from *P. gyrans* and *D. salina* inhibited ACE between 21.7% and 37.9% at concentrations of 4 mg mL^{-1} (Figure 7a). Abz–Gly–Phe(NO2)–Pro (4 mg mL^{-1}) was used as a positive control, which showed a capacity to inhibit ACE of 39.9 ± 0.5%. The aqueous extracts of both microalgae exhibit a high ACE inhibition potential. In addition, *P. gyrans* extracts showed higher efficiency than *D. salina* extracts. Indeed, the *P. gyrans* aqueous extracts showed a similar ability to inhibit ACE as observed in the positive control tested. These results are in line with the literature, given that the aqueous extracts had a higher protein content (in terms of peptides and amino acids), with *P. gyrans* presenting a higher and more diverse protein fraction than *D. salina*.

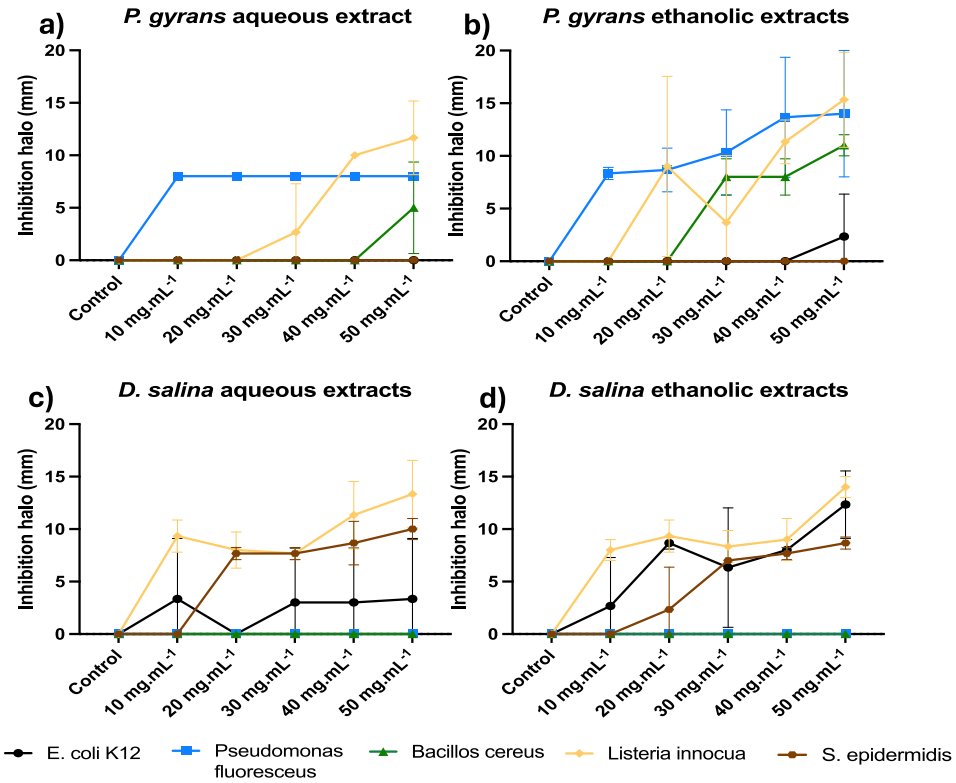

FIGURE 6 | Antibacterial activity of *P. gyrans* and *D. salina* aqueous and ethanolic extracts with different concentrations (10, 20, 30, 40, and 50 mg mL^{-1}) against Gram-positive (*Bacillus cereus*, *Staphylococcus epidermidis*, and *Listeria innocua*) and Gram-negative (*Escherichia coli* K12 and *Pseudomonas fluorescens*) bacteria. Data is presented as mean ± standard error of the mean.

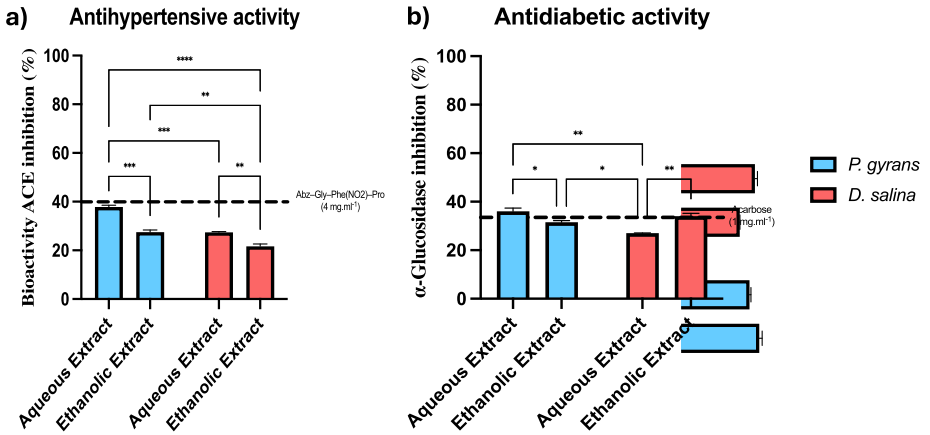

FIGURE 7 | Antihypertensive activity (ACE inhibition) (a) and antidiabetic activity (α-glucosidase inhibition) (b) induced by the aqueous and ethanolic extracts of *D. salina* and *P. gyrans* at a concentration of 4 mg mL^{-1}. Positive controls for both assays are included for comparison, while negative controls were previously subtracted from the experimental conditions. Data is presented as mean ± standard error of the mean. Statistical significance was considered for (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, and (****) $p \leq 0.0001$.

The small intestine epithelial cell brush border contains an oligosaccharide hydrolase, α-glucosidase, which hydrolyses oligosaccharides and disaccharides into monosaccharides [52]. The inhibition of α-glucosidase delays carbohydrate breakdown and glucose absorption, helping to control postprandial hyperglycemia and providing an effective strategy for managing type 2 diabetes mellitus [10, 23]. Evaluating the activity of specific enzymes responsible for sugar metabolism allows inferring the antidiabetic power of extracts and compounds. In this regard, the screening of antidiabetic activity has been performed for the aqueous and ethanolic extracts from both microalgae by evaluating their inhibition power of α-glucosidase at concentrations of 4 mg mL^{-1} (Figure 7b). The present study demonstrated that all the extracts tested had an α-glucosidase inhibitory effect between 27% and 36%. Acarbose (1 mg mL^{-1}) was used as a positive control, which showed a capacity to

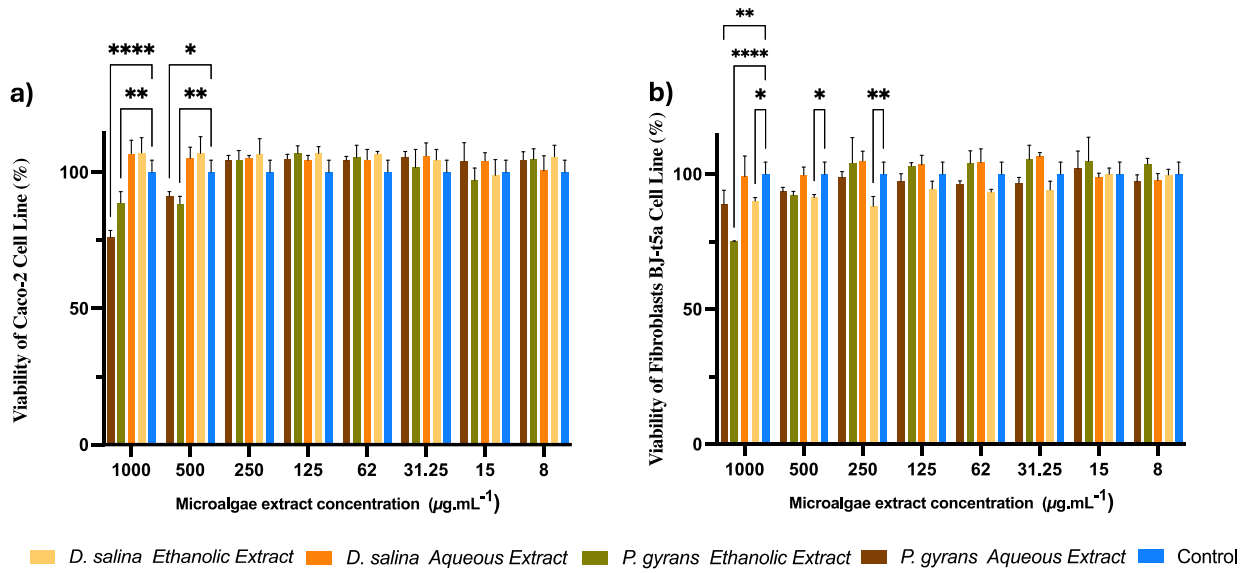

FIGURE 8 | Effect of *P. gyrans* and *D. salina* aqueous and ethanolic extracts with different concentrations on viability of Caco-2 (a) and Bj5ta cells (b). Data is presented as mean ± standard error of the mean. Statistical significance between the sample and the control was considered for (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, and (****) $p \leq 0.0001$.

inhibit α-glucosidase of 33.5 ± 0.5%. In fact, the *P. gyrans* aqueous extracts and the *D. salina* ethanolic extracts showed higher ability to inhibit α-glucosidase than the use of 1 mg mL^{-1} of acarbose. Previous studies showed that eight photobiont extracts from the *Coelastrella* and *Chlorella* genus present a maximum α-amylase inhibitory rate near 30% at a concentration of 1-10 mg mL^{-1} [10]. Similar findings were reported for the polyphenolic extract from sugarcane straw with α-glucosidase inhibitory effect between 15% and 38% at an extract concentration of 1.25–5.00 mg mL^{-1} [23]. All extracts from *D. salina* and *P. gyrans* biomass showed α-glucosidase inhibitory capacity similar to those described above, reinforcing that they possessed moderate α-glucosidase inhibitory activity. Several biocompounds present in microalgae biomass have been suggested as displaying antidiabetic activity, such as pigments, peptides, fatty acids or phenolic compounds [6, 7, 23]. Based on the extract composition determined in the present study, several of those compounds were detected which could be connected to the antidiabetic activity observed.

3.9 | Cell Viability

The effect of *P. gyrans* and *D. salina* aqueous and ethanolic extracts on cell viability of Caco-2 and Bj5ta cell lines is presented in Figure 8. The *D. salina* extracts did not have a significant effect on Caco-2 cell viability ($p > 0.05$) compared to the control. On the other hand, both *P. gyrans* extracts significantly reduced the Caco-2 viability ($p < 0.05$) at concentrations of 1000 and 500 μg mL^{-1}. Regarding the Bj5ta fibroblast cell line, the cells showed a behavior similar to that obtained in Caco-2, since both *P. gyrans* extracts concentration at 1000 μg mL^{-1} showed a significant cell viability difference compared to the control ($p < 0.05$). However, the *D. salina* ethanolic extract significantly reduced Bj5ta viability ($p < 0.05$) at concentrations of 1000, 500, and 250 μg mL^{-1}. Many studies have shown that a compound is considered non-toxic if the cell viability exceeds 70% [44, 53]. Despite the differences observed in Figure 8, none of the extracts in the study, in the range evaluated, contributed to cell viability lower than 70%. Additionally, it was suggested that an IC_{50} value (lethal concentration for 50% of the cells) lower than 100 μg mL^{-1} suggests that an extract has a significant cytotoxic effect [44, 54]. The IC_{50} values of the *D. salina* and *P. gyrans* extracts were higher than 100 μg mL^{-1}, which could indicate that the extracts were not cytotoxic to the cell lines studied. Based on these assumptions, the MTT assay confirmed that cells treated with both *D. salina* and *P. gyrans* extracts were not cytotoxic for Caco-2 and Bj5ta cell lines for concentrations up to 1000 μg mL^{-1}. Human cells utilize the antioxidant properties of some metabolites from microalgae to maintain or enhance their viability [42]. As previously stated, the extracts generated in the current study had a significant CAA (Figure 5) due to the presence of bioactive compounds such as carotenoids, peptides, or phenolic compounds, which protected cells from oxidative stress and enhanced cell growth. The results reported by Senhorinho et al. [55] align closely with the findings of our study. Specifically, the non-tumorigenic mammary epithelial cell lines (MCF10A and 184B5) did not exhibit any reduction in cell viability when exposed to extracts from five wild *Chlamydomonas* species, *Chlamydomonas reinhardtii*, and *Scenedesmus dimorphus* at concentrations ranging from 5 to 150 μg mL^{-1}. Instead, an increase in viability was observed at certain extract concentrations, particularly in the 184B5 cell line. These results underscore the potential of these algal extracts to positively influence cell viability.

3.10 | Multivariate Analysis

The PCA analysis revealed a clear differentiation between samples, with the first two principal components (PC1 and PC2) explaining 46.8% and 27.9% of the variance, respectively, accounting for 74.7% of the total variability (Figure 9). This high cumulative variance indicates that the principal components adequately represent the underlying data structure. The ellipses,

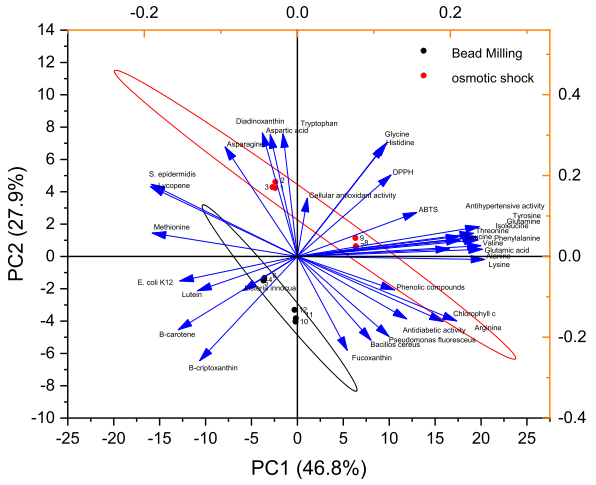

FIGURE 9 | PCA of the data obtained regarding the bioativities of the extracts produced from microalgae biomass of *P. gyrans* and *D. salina* regarding the type of treatment applied.

formed with a 95% confidence interval, demonstrate that both bead milling and osmotic shock methods yield distinct results, independent of the microalgae species used. Osmotic shock included extracts from samples 1, 2, 3, 7, 8, and 9, while bead milling comprised extracts from samples 4, 5, 6, 10, 11, and 12. The choice of water (osmotic shock) and ethanol (bead milling) as extraction solvents was intended to target compounds of different polarity. Water was selected to recover hydrophilic components, such as small peptides and amino acids, whereas ethanol favors lipophilic pigments extraction, such as carotenoids and chlorophyll derivatives [9, 34, 35]. Similar solvent systems are widely used in the extraction of bioactive molecules from microalgae for functional applications [10, 16, 27]. Nevertheless, it should be noted that these solvents may also partially dissolve polysaccharides and other macromolecules (not evaluated in the present study) that may also have an impact on the assessed bioactivities. The separation of the two groups highlights distinct biochemical and functional profiles, particularly driven by variables such as β-carotene, lutein, and β-cryptoxanthin, which were strongly associated with samples along the negative y-axis, while asparagine and diadinoxanthin were more prominent along the positive y-axis. Within the same clusters, samples were also separated according to the microalgae species: *P. gyrans* extracts (7 to 12) exhibited a greater variety of amino acids as well as antidiabetic, antioxidant, and antihypertensive activities, whereas *D. salina* extracts (1 to 6) presented higher levels of β-carotene, lutein, β-cryptoxanthin, and fucoxanthin. The alignment of antioxidant activities (DPPH and ABTS) and amino acids (e.g., glycine, glutamic acid, threonine, isoleucine, among others) in the upper-right quadrant suggests correlated functional properties. From a structural–functional perspective, the distinct peptide and pigment profiles of both microalgae appear to play a central role in their bioactivity. Specifically, the aqueous extracts of *P. gyrans* were rich in low–molecular-weight peptides (<3–5 kDa) and amino acids such as glutamic acid, aspartic acid, leucine, and alanine. These residues were associated with antioxidant and antihypertensive properties in the PCA analyses. Indeed, previous studies have shown that small peptides containing hydrophobic or aromatic amino acids display strong radical-scavenging and ACE-inhibitory effects [15–18]. On the other hand, the radical-quenching potential (antioxidant activity) observed in the ethanolic extracts could be associated with the higher concentrations of xanthophyll carotenoids, particularly fucoxanthin (in the case of *P. gyrans*) and lutein (in *D. salina*). These carotenoids possess extended conjugated double-bond systems and oxygenated end groups, which enhance electron donation and contribute to the antioxidant potential of the ethanolic extracts [56, 57]. The bead milling method appears to be associated with the release of compounds such as β-carotene, lutein, and β-cryptoxanthin, while osmotic shock is more closely related to higher concentrations of amino acids and antihypertensive and antidiabetic activities as indicated by variable vectors in Figure 9. This differentiation can be attributed to the physical factors involved in cell disruption as well as the nature of the solvent used. The complementary bioactivities observed between the different extraction solvents and techniques reflect the effectiveness of the dual extraction approach. Aqueous fractions favored the recovery of polar compounds (amino acids, small peptides) whereas ethanolic fractions concentrated pigments. Such complementarity suggests potential synergistic effects when both fractions are used together, aligning with the growing interest in whole-extract applications for functional and nutraceutical formulations.

4 | Conclusions

This study demonstrated that both ethanolic and aqueous extracts from the marine microalgae *Dunaliella salina* and *Pavlova gyrans* exhibit notable bioactivities, including antioxidant, antibacterial, antidiabetic, and antihypertensive effects, while maintaining a favorable safety profile in human cell lines (Caco-2 and BJ5ta) at concentrations up to 1000 µg mL^{-1}. The distinct biochemical compositions of the extracts—such as the higher carotenoid content in *D. salina* and the broader amino acid profile in *P. gyrans*—contribute to their differentiated functional properties, with *P. gyrans* extracts showing enhanced antidiabetic, antioxidant, and antihypertensive potential. These findings support the viability of microalgae-derived extracts as sustainable, health-promoting ingredients for future food systems. By combining demonstrated bioactivity with safety, these extracts represent promising candidates for the development of functional ingredients, aligned with current challenges in food innovation, nutrition, and sustainability.

Acknowledgments

This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic funding of the UIDB/04469/2020 unit, with DOI https://doi.org/10.54499/UIDB/04469/2020, and by LABBELS—Associate Laboratory in Biotechnology, Bioengineering and Microelectromechanical Systems, LA/P/0029/2020. Vitor Sousa acknowledges FCT for his fellowship UI/BD/151238/2021 (https://doi.org/10.54499/UI/BD/151238/2021). This work was financially supported by "Pacto da Bioeconomia azul" (Project No. C644915664-00000026) within the WP5 Algae Vertical, funded by Next Generation EU European Fund and the Portuguese Recovery and Resilience Plan (PRR), under the scope of the incentive line "Agendas for Business Innovation" through the funding scheme C5 - Capitalization and Business Innovation. Pedro Geada acknowledges FCT for the Junior Research contract obtained under the scope of the Scientific Stimulus Employment

with the ref. 2022.00930.CEECIND (https://doi.org/10.54499/2022.00930.CEECIND/CP1718/CT0023).

Funding

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data available on request from the authors

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File: mnfr70398-sup-0001-SupMat.docx.