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Nonthermal high-pressure microalgae extracts: A new source of natural ingredients for cosmetics

Mónica P. Garcia [a,b], Ana Regueiras [c], Graciliana Lopes [c], Gabriela Matos [d], Lucília P. da Silva [a,b], Mariana T. Cerqueira [a,b], Helena Cardoso [e], Nádia Correia [e], Jorge A. Saraiva [d], Joana L. Silva [e], Rosário Martins [c,f], Alexandra P. Marques [a,b,*]

[a] 3B's Research Group, I3Bs - Research Institute on Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Parque de Ciência e Tecnologia, Zona Industrial da Gandra, 4805-017 Barco, Guimarães, Portugal
[b] ICVS/3B's - PT Government Associate Laboratory, Braga, Guimarães 4805-017, Portugal
[c] CIIMAR-Interdisciplinary Centre of Marine and Environmental Research, Matosinhos, Portugal
[d] LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal
[e] Allmicroalgae Natural Products S.A., R&D Department, Rua 25 de Abril 19, 2445-287 Pataias, Portugal
[f] CISA-ESS/PORTO, Health and Environment Research Centre, School of Health, Polytechnic Institute of Porto, Porto, Portugal

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ABSTRACT

Microalgae are one of the most prominent sources of ingredients for the cosmetic industry not only due to their diversity but, importantly, due to the low environmental impact in production and extraction. A sustainable extraction process requires the use of effective and environmentally friendly solvents that guarantee an extract with a relevant content and in high yields. In this work, *Chlorella vulgaris*, *Nannochloropsis oceanica*, *Tetraselmis chui*, *Chlorococcum amblystomatis* and *Phaeodactylum tricornutum* microalgae species grown in tubular photobioreactors were used to validate a high-pressure extraction (HPE) method. Extraction yields up to 57 % were obtained depending on the used solvent (water, ethanol, or acetone). The extracts were then characterized regarding their phytochemical composition (total phenolic, protein, chlorophylls content, and chlorophyll *a*). Aqueous extracts showed high protein content (6–51 μg/mg biomass), while ethanolic and acetone extracts showed high amounts of phenolic compounds (0.0007–0.03 μg/mg biomass). The levels of photosynthetic pigments (0.1–11 μg/mg biomass) associated with antioxidant and anti-inflammatory properties were higher when extracted using ethanol and high-pressures. *Chlorella vulgaris* aqueous extracts presented higher protein content while *Nannochloropsis oceanica* and *Tetraselmis chui* ethanolic extracts presented higher amounts of photosynthetic pigments. The effect of the extracts over the metabolic activity of primary human dermal fibroblasts, keratinocytes, melanocytes, and adipocytes was dependent on both microalgae species and cell type. In adipocytes and fibroblasts, extracts presented an IC50 > 500 μg/mL, except for the one of *Phaeodactylum tricornutum* in fibroblasts (IC50 < 150 μg/mL). In contrast, the IC50 was below 500 μg/mL for most of the extracts in keratinocytes and melanocytes. Overall, extracts of sustainably-grown microalgae obtained by a high-pressure method are a promising source of natural ingredients for cosmeceutical applications.

1. Introduction

Microalgae are in the spotlight of the cosmetic industry for both the production of bioactive compounds and for the sustainability in biomass production and extraction. Microalgae are rich in bioactive compounds such as proteins, carbohydrates, lipids, pigments, vitamins and minerals

* Corresponding author at: 3B's Research Group, I3Bs - Research Institute on Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Parque de Ciência e Tecnologia, Zona Industrial da Gandra, 4805-017 Barco, Guimarães, Portugal.

E-mail addresses: aregueiras@ciimar.up.pt (A. Regueiras), glopes@ciimar.up.pt (G. Lopes), gabrielamatos@ua.pt (G. Matos), lucilia.silva@i3bs.uminho.pt (L.P. da Silva), helena.cardoso@allmicroalgae.com (H. Cardoso), jorgesaraiva@ua.pt (J.A. Saraiva), joana.g.silva@allmicroalgae.com (J.L. Silva), mrm@ess.ipp.pt (R. Martins), apmarques@i3bs.uminho.pt (A.P. Marques).

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that can confer specific properties to cosmetics, such as sunscreens, moisturizers, and anti-aging products due to their antioxidant, immune-stimulating, anti-inflammatory, anti-microbial, anti-viral, and anti-fungal properties [1,2]. As examples, microalgae protein hydrolysates were shown to enhance the production of extracellular matrix (ECM) components while diminishing ECM-degrading enzymes, conferring firmness to the skin and reducing wrinkle appearance [3]. Components with UV light absorbance capability, such as mycosporine-like amino acids (MAA) and pigments (β-carotene, fucoxanthin, astaxanthin) are used as filters in sunscreens [4]. Moreover, pigments *per se* possess strong anti-oxidant and anti-microbial properties relevant for providing skin protection against UV radiation and microbial invasion, respectively [5]. Similarly, microalgae lipid extracts were found to inhibit the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), and reactive oxygen species (ROS) by human peripheral blood mononuclear cells [6].

Concerning biomass production microalgae are also a sustainable solution with low environmental impact since they can be grown without the use of valuable resources such as fresh water or arable land, they use sunlight as an energy source, capture CO_2 and have high photosynthetic yield [7]. Additionally, microalgae can be cultivated in photobioreactors, where the abiotic conditions can be modified according to the expected application, increasing productivity or composition [5]. The sustainability of microalgae-based ingredients can be further improved by innovative extraction technologies such as high-pressure extraction (HPE). Conventional technologies based on solvent extraction have limitations such as low extraction yield and selectivity, and the use of high quantities of solvents when compared to high-pressure technologies [8]. In turn, HPE has been recognized as an environmentally friendly technology by the FDA, since it does not produce effluents and requires low energy consumption [9]. Moreover, HPE is a non-thermal extraction methodology, as it can operate at room temperature, with low to no impact in the activity of heat-sensitive compounds. It is also associated with short-time processing, high solubility, and consequent high extraction yield [9].

Microalgae species in commercial products or with commercial potential include *Chlorella vulgaris*, *Tetraselmis chui*, *Nannochloropsis oceanica*, *Phaeodactylum tricornutum*, and *Chlorococcum amblystomatis* [4]. *C. vulgaris is* composed of 42–58 % of proteins, 5–40 % lipids, 11–55 % carbohydrates and pigments (1–2 % chlorophyll) and minerals [10] has been previously linked to antioxidant, anti-inflammatory, wound healing, anticancer, antiacne, anti-obesity, and antibacterial properties [6,11–15]. In dermal fibroblasts, *C. vulgaris* extracts demonstrated anti-aging effects by reducing DNA damage, telomere shortening and telomerase activity [16]. *C. vulgaris*-derived peptides have also been shown an to have anti-photoaging effect in UVB-irradiated fibroblasts by inhibiting the expression of matrix metalloproteinase-1 (MMP-1) which contributes to collagen degradation, while also restoring the expression of procollagen [17]. Moreover, *C. vulgaris*-derived peptides also inhibited the cytotoxicity induced by UVC in fibroblasts by inhibiting the caspase-3 apoptotic pathway as shown by reduced phosphorylated FADD and cleaved PARP-1 [18]. *T. chui* holds cosmeceutical potential due to a high content of carbohydrates (31 %), α-tocopherol and eicosapentaenoic acid [19]. It has shown antioxidant activity due to the free radical scavenging capacity of phenolic compounds, pigments, and lipids present in aqueous, organic and lipid-rich extracts [20,21]. However, its effects over skin cells have not been studied yet. *N. oceanica*, *P. tricornutum*, and *C. amblystomatis* are not commonly used in cosmetic products, despite presenting great potential to be used as cosmeceuticals. *N. oceanica* has a high content of lipids (21 %) (especially eicosapentaenoic acid) and pigments (such as violaxanthin), components which have been linked to antioxidant properties [21–23]. *N. oceanica* pigmented extracts, containing violaxanthin, were also reported to have a protective effect on fibroblasts exposed to UV light, downregulating the expression of metalloproteinases, increasing the expression of collagen, and decreasing the production of ROS [23]. *P. tricornutum* extracts rich in fucoxanthin and *P. tricornutum*-derived fucoxanthin were shown to have antioxidant and anti-obesity activity [24,25]. Moreover, *P. tricornutum* extracts have also been reported to have a protective effect in keratinocytes exposed to UV light by preserving the proteasome activity, thus decreasing the extent of protein oxidative damage [26]. The *C. amblystomatis* lipid extracts, 18–32 % of the content with a high content of omega-3 polyunsaturated fatty acids (PUFAs) [27,28], have anti-inflammatory effects by decreasing nitric oxide production in RAW264.7 cell line, and decreasing COX-2 activity [13,27]. Moreover, these extracts have shown antioxidant effects, due to their free radical scavenging capacity [27].

Led by the described bioactive features, herein we aimed to explore a high-pressure extraction method as a sustainable process to obtain microalgae extracts with cosmeceutical potential. Hence, aqueous and organic extracts were obtained varying different parameters (solvent, pressure, time) and the optimal conditions were defined based on the process yield and phytochemical profile (total phenolic content (TPC), total protein content, total chlorophyll *a*nd chlorophyll *a* content). Moreover, cytotoxicity analysis in different skin cells was performed identifying the IC50 of each extract for each cell type.

2. Materials and methods

2.1. Biomass production

Tubular photobioreactors were inoculated using Allmicroalgae's base medium (based on Guillard's F/2 medium) with a final nitrogen concentration of 10 mM as described by Guerra *et al.* (2021) [29]. In saltwater species (*N. oceanica*, *T. chui*, and *P. tricornutum*) medium was supplemented with $30 gL^{-1}$ of NaCl (Salexpor, Coimbra, Portugal) and a magnesium-enriched supplementation (Necton, Portugal). Cultures were kept outdoors, subjected to natural circadian light:dark cycles with approximately 16 h of light per day, with pH control through pure CO_2 injection and continuous aeration. Biomass growth was monitored by optical density measurements at 600 nm using Genesys 10S UV–Vis spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

2.2. Microalgae extract preparation

Extracts were obtained by HPE by mixing 125 mg of dried microalgae with 25 mL of extraction solvent (water, ethanol 48 % and 96 % or acetone). The mixture was then placed in low permeability polyamide–polyethylene bags (PA/PE-90, Ideiapack, Viseu, Portugal). HPE was performed in a 55 L capacity industrial-scale high-pressure equipment (model 55, Hiperbaric, Burgos, Spain), in a pressure range of 100–500 MPa, and carried out at ambient temperature (~20 °C), up to a maximum of 15 min, using water as a pressure-transmitting medium (all extraction conditions were performed in triplicate for each microalgae species). Control samples were maintained at atmospheric pressure (0.1 MPa) under the same conditions of time and solvent. After extraction, samples were centrifuged at 15000 rpm for 10 min at 4 °C, filtered with standard filter paper (ST61 150 mm, JMGS, Odivelas, Portugal) and collected, followed by freeze-drying or evaporation (for corresponded water or ethanol solvents) to obtain a dry powder extract. The extraction yield was determined as the amount of extract obtained per 1 g of microalgae dried weight.

2.3. Microalgae extract yield optimization

To determine the optimal extraction conditions, maximizing extraction yield, the Response Surface Methodology (RSM) of Minitab Statistical Software was used, followed by a central composite face-centered design (CCD). Previous studies regarding the use of HPE for the extraction of bioactive compounds allowed to choose three independent factors as potential variables affecting extraction: pressure level (P: 100–500 MPa), holding time (t: 1–15 min) and solvent concentration

(S: 0–100 % ethanol, *v*/v) [9]. Each variable to be optimized was evaluated at three levels: −1, 0, +1. The response variable was extraction yield (% m/m). Twenty random experimental runs were performed in total. Each point was carried out in triplicate and the central point was duplicated.

The relationship between the response and the independent factors in the CCD follows a second-order polynomial model:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{j>1}^{k} \beta_{ij} X_i X_j$$

where Y is the predicted dependent variable, X_i and X_j are the independent variables, and k is the number of tested variables. Model coefficients are defined as β_0 for the intercept, β_i for linear terms, β_{ii} for quadratic terms, and β_{ij} for interaction terms. Only the statistically significant terms ($p < 0.15$) were considered to simplify the models by a stepwise procedure. Analysis of variance (ANOVA) was carried out to determine the F-value for the significant ($p < 0.05$) linear, quadratic, and interaction effects. Finally, the optimal extraction conditions and the consequent maximum value were predicted. A new assay was performed, and the obtained experimental values were compared to the predicted ones, validating the predicted values by the statistical model in terms of percentage.

2.4. Chemical profiling of microalgae extracts

2.4.1. Total phenolic content (TPC)

The TPC of extracts was determined using the colorimetric assay based on the Folin-Ciocalteu method, according to Barroso *et al.* (2016) with some modifications [30]. Briefly, a volume of 25 μL of each extract (10 $mgmL^{-1}$) was thoroughly mixed with 25 μL of Folin-Ciocalteu reagent (Sigma-Aldrich), 200 μL of Na_2CO_3 solution (75 gL^{-1}) and 500 μL of deionized water. After 60 min of incubation, absorbance was measured at 725 nm, using a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by GEN5TM software. Each time the TPC were determined a standard calibration curve was obtained with seven concentrations of gallic acid (GA) (from 0.015625 to 1 mg mL^{-1}). TPC was expressed as μg GA equivalents (GAE) per mg of dry extract. Each measurement was carried out in triplicate.

2.4.2. Total protein content

Total proteins were quantified using the PierceTM BCA protein assay (#23227 ThermoFisher Scientific), as described by the manufacture company. Aqueous extracts were prepared in water and acetone and ethanolic extracts in DMSO. A standard calibration curve was obtained with BSA (bovine serum albumin) (from 0,03125 to 1 $mgmL^{-1}$) each time the quantifications were performed. Briefly, a volume of 25 μL extract was mixed with 200 μL of working reagent in a 96 well plate. Plates were shaken for 30 s, incubated for 30 min at 37 °C (in the dark) and let to cool at room temperature. The absorbance was measured at 562 nm, using a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by GEN5TM software. The total protein content was expressed as μg of bovine serum albumin (BSA) equivalents per mg of dry extract. Each measurement was carried out in triplicates.

2.4.3. Chlorophyll a and total chlorophyll contents

Chlorophyll a and total chlorophylls existing in aqueous, ethanol and acetone extracts were quantified spectrophotometrically, by measuring the absorbances, at 663 nm and 645 nm using a Synergy HT Multi-detection microplate reader (Biotek, Bad Friedrichshall) operated by GEN5TM software. Pigments were determined by applying the formulas:

$$\text{Chlorophyll a (Chla)} = 12.7^{*}A663\ \text{nm} - 2.69^{*}A645\ \text{nm}$$

$$\text{Total chlorophyll (TChl)} = 20.2^{*}A645\ \text{nm} + 8.02^{*}A663\ \text{nm}$$

2.5. Cell culture

Human dermal fibroblasts (hDFBs) and keratinocytes (hKCs) were isolated from skin specimens of healthy donors undergoing abdominoplasties as described by Cerqueira *et al.* (2014) [31]. Skin samples were obtained after informed consent and under the protocol established and approved by the Ethical Committee of Hospital S. João (Porto, Portugal) (Nr 477/2020) and the *Comissão de Ética para a Investigação em Ciências da Vida e da Saúde* (Braga, Portugal) (Nr 135/2020). Fibroblasts were cultured in α-MEM supplemented with 10 % of fetal bovine serum (FBS, Gibco, Life Technologies, Netherlands) and 1 % of antibiotic/antimycotic solution (Gibco, Life Technologies, Netherlands). Keratinocytes were cultured in FAD medium (Dulbecco's modified Eagle's medium: HAM's F12 medium (Sigma, USA) [3:1] supplemented with 10 % non-inactivated FBS (Gibco, Life Technologies, Netherlands), 5 μgmL^{-1} insulin (Sigma, USA), 1.8×10^{-4} M adenine (Sigma, USA), 0.5 μgmL^{-1} hydrocortisone (Sigma, USA), 10^{-10} M cholera toxin (Sigma, USA), 10 $ngmL^{-1}$ epidermal growth factor, 1.8 mM $CaCl_2$ (Sigma, USA), and 1 % penicillin-streptomycin (Life Technologies, Netherlands), in an 3T3-J2 fibroblast feeder monolayer previously inactivated with 4 μgmL^{-1} of mitomycin (Sigma, St. Louis, MO) [32]. For experiments, hKCs were cultured in Keratinocyte - serum free medium (K-SFM, Gibco, Life Technologies, Netherlands). Melanocytes (hMels) were purchased from Cell Applications Inc. (neonatal foreskin hMel, catalog no.104-05n, USA) and were cultured in HEM Growth Medium Kit (Cell Applications, Inc., USA). Pre-adipocytes (PAdip) were purchased from Lonza (Switzerland) and were cultured in PGM-2™ Preadipocyte Growth Medium-2 BulletKit (Lonza, Switzerland). Cell cultures were maintained under standard conditions (37 °C and 5 % CO_2) in a humidified atmosphere and medium was changed every 2–3 days, according to cell culture specifications. Cells were used between passages 2 and 5 in the experiments.

2.6. Citotoxicity assay

Cells were cultured at a density of 3×10^4 $cell/cm^2$ in 96-well plates. After the first 24 h, extracts were added at a concentration of 500, 100 and 10 μgmL^{-1}, except for the pre-adipocytes that were cultured in PGM-2™ differentiation medium for 6 days prior extracts supplementation. Extract dilutions were prepared from an aqueous (water extracts) or 96 % ethanol (ethanolic extracts) stock solution (50$mgmL^{-1}$) using culture medium. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega, USA) was used according to manufacture instructions after 3 days of incubation with the extracts. The absorbance was read at 490 nm in a microplate reader (Synergy HT, BIO-TEK, USA). Results are presented as % relative to control cultures (maintained in standard conditions with 1 % solvent (1 % water or 1 % ethanol 96 % in culture medium). A positive control (10 % DMSO in culture medium) for cytotoxicity was included. The 50 % inhibitory concentration (IC50) was calculated from the cytotoxicity results and determined by non-linear regression curve fit in GraphPad Prism software version 7.04.

2.7. Statistical analysis

Statistical analyses of the cytotoxic evaluation were performed in GraphPad Prism 7.04 software. The results were tested for normality by D'Agostino & Pearson normality test. Non-parametric data were evaluated using Kruskall-Walli's test followed by Dunns' post-test, with a significance value at $p < 0.05$. Data are presented as mean ± standard deviation, from 3 independent experiments ($n = 3$) with 3 replicates for each condition.

3. Results

3.1. Microalgae biomass production

The microalgae species *C. vulgaris*, *T. chui*, *N. oceanica*, *P. tricornutum*, and *C. amblystomatis* were scaled-up and locally produced, in Allmicroalgae's photobioreactors. Overall the highest productivity was 0.261 g/L/day for *C. vulgaris*, 0.304 g/L/day for *T. chui*, 0.107 g/L/day for *N. oceanica*, 0.159 g/L/day for *P. tricornutum* and 0.083 g/L/day for *C. amblystomatis*.

3.2. Microalgae extraction yield

C. vulgaris was used in the preliminary tests to determine the best biomass/solvent ratio, under HPE extraction conditions previously used for different herbal materials [9]. Extraction time between 1 and 15 min, pressure intensity set between 100 and 500 MPa and distilled water, 96 % ethanol (*v*/v) and 48 % ethanol (v/v) as extraction solvents, were selected. The optimal percentage of biomass at defined as 0.5 %. To maximize the yield, statistical optimization of extraction conditions of *C. vulgaris* was performed by Response Surface Methodology (RSM) followed by central composite face-centered design (CCD). Fig. 1 displays the extraction yield (%m/m) results for *C. vulgaris*, categorized by extraction solvent, corresponding to distinct extraction conditions (MPa/min). The statistical model estimated the optimum conditions for the extraction yield (%m/m) of *C. vulgaris* as follows: 420 MPa of pressure intensity, 9 min of extraction time and water as the extraction solvent. These conditions corresponded to a maximum predicted yield value of 16.75 %, for *C. vulgaris*. When the predictive optimal conditions were used experimentally, a yield of 16.30 ± 0.27 % was obtained which validates the result of the statistical model.

Considering the HPE conditions and results described for *C. vulgaris*, studies with *N. oceanica*, *C. amblystomatis*, *P. tricornutum* and *T. chui* were conducted, using the same conditions with the purpose to compare the results with *C. vulgaris*. Additionally, and taking into consideration the morphology and composition of each of the microalgae [33], two other extraction conditions were used (one more intense and the other less intense than the optimum obtained for *C. vulgaris*). For *N. oceanica*, *C. amblystomatis* and *P. tricornutum* conditions were set at (i) 500 MPa during 15 min (ii) 300 MPa during 15 min and, using distilled water, 96 % ethanol (*v*/v), 48 % ethanol (v/v) and also acetone, as extraction solvents. For *T. chui*, that has thinner [34,35] and thus weaker cell wall, pressure-time conditions were not as intense and were defined at 300 and 100 MPa during 9 min. Fig. 2 shows the average extraction yields % (m/m), grouped by extraction solvent, for each of the HPE conditions for *N. oceanica*, *C. amblystomatis*, *P. tricornutum* and *T. chui* microalgae, as well as the controls at atmospheric pressure (0.1 MPa). The highest extraction yields, between 13 and 17 %, were found when water was used as solvent while the lowest yields were obtained with 96 % (v/v) ethanol. Furthermore, a trend of increasing extraction yield was observed with increasing pressure intensity (from 100 MPa to 500 MPa) while yield was independent of the time of extraction.

3.3. Phytochemical composition of the extracts

Microalgae extracts were analyzed in terms of phytochemical composition to understand the effect of the different extraction conditions on the TPC, protein content, total chlorophyll *a*nd chlorophyll *a* content (Table 1, Supplementary Table 1). For *C. vulgaris*, the extract obtained with water at 420 MPa for 9 min was the one with higher amount of both proteins (55.180 ± 1.951 mg BSA/mg dry weight), chlorophyll *a* (363.079 ± 106.237 μg/mg dry weight), and TPC (0.004 μg GAE/mg dry weight). Concerning *N. oceanica*, TPC ranged from 0.006 μg GAE/mg (48 % ethanol and at 500 MPa for 15 min) to 0.001 μg GAE/mg dry weight (most of the other conditions and controls). The highest concentration of protein (14.950 ± 1.237 mg BSA/mg dry weight), chlorophyll *a* (887.988 ± 94.147 μg/mg dry weight) and total chlorophylls (1087.020 ± 134.320 μg/mg dry weight), was attained with

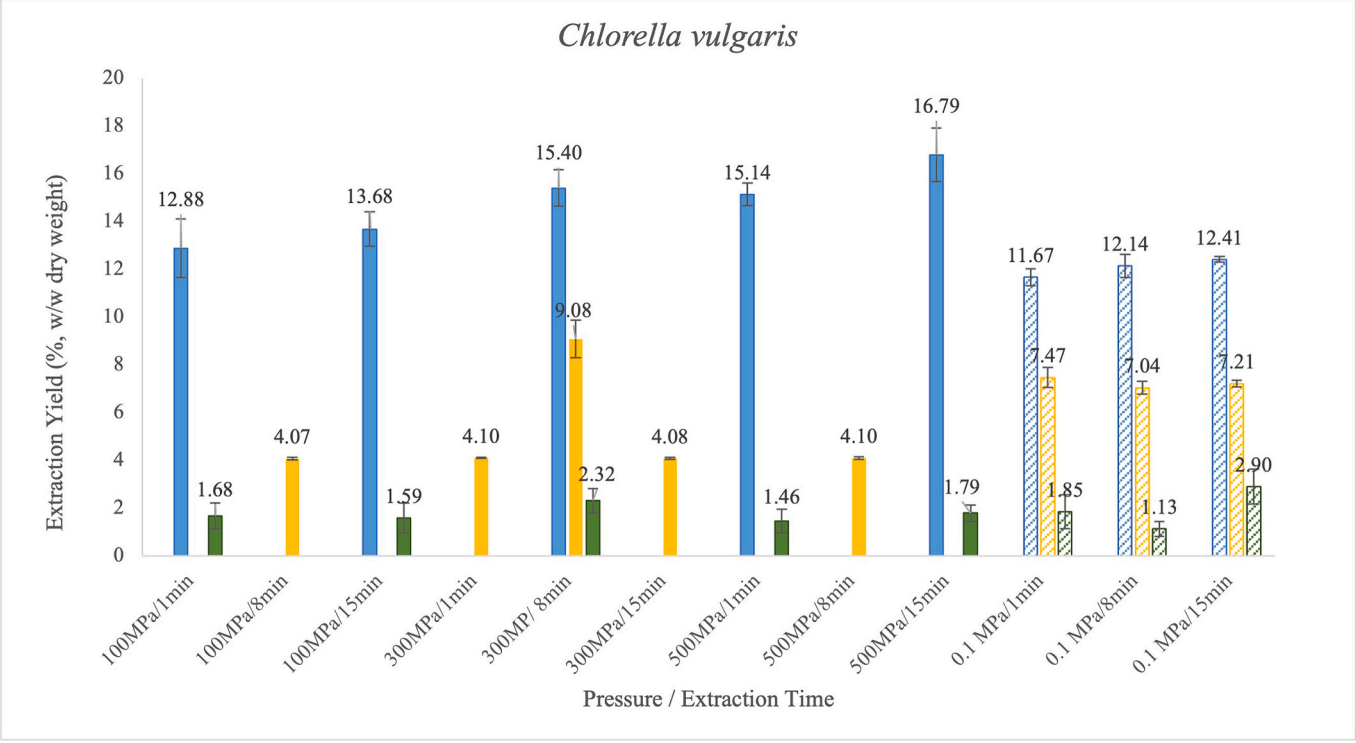

Fig. 1. *C. vulgaris* average extraction yields % (m/m) with varied pressure and time, grouped by extraction solvent. Controls are represented by dashed lines, each corresponding to the color of its respective solvent. Results are expressed as mean ± SD of three independent extractions. Data used for statistical optimization of extraction conditions of *C. vulgaris*.

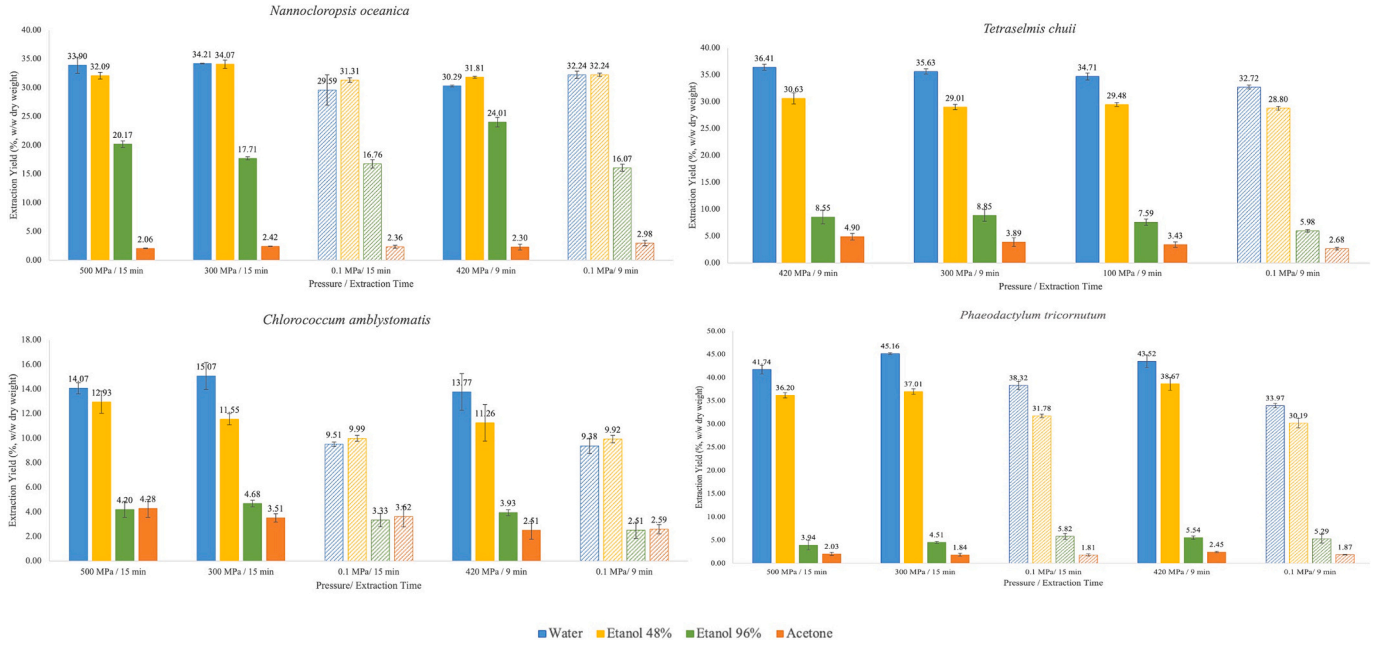

Fig. 2. *N. oceanica*, *C. amblystomatis*, *P. tricornutum* and *T. chui* average extraction yields % (m/m) with varied pressure and time, grouped by extraction solvent. Controls are represented by dashed lines, each corresponding to the color of its respective solvent. Results are expressed as mean ± SD of three independent extractions.

water at 500 MPa for 15 min. The *P. tricornutum* extract at 420 MPa for 9 min was the one with higher TPC content, proteins and chlorophyll. For chlorophylls, the higher concentration was obtained with ethanol at 96 % as solvent (331.578 ± 3.260 μg/mg dry weight and 406.179 ± 6.452 μg/mg dry weight for chlorophyll *a* and total chlorophylls respectively). However, for total protein and phenolic compounds, the extract obtained with ethanol 48 % was the one with higher content, respectively 26.295 ± 2.087 μg BSA/mg dry weight and 0.057 ± 0 μg GAE/mg dry weight. For the microalga *T. chui* the highest TPC (0.0355 ± 0 μg GAE/mg dry weight) was detected in the extract obtained with the acetonic extraction at 420 MPa for 9 min. In turn, the extracts obtained at 300 MPa for 9 min with ethanol at 96 % had the highest total protein (10.006 ± 1.407 of μg BSA/mg dry weight), chlorophyll *a* (887.988 ± 94.105 μg/mg dry weight) and total chlorophyll (887.988 ± 94.105 and 1087.020 ± 134.320 μg/mg) content.

Taking in consideration these data, the HPE conditions resulting in microalgae extracts with higher content of bioactive components and high extraction yield were selected for subsequent analysis, *i.e.* water/420 MPa/9 min and ethanol 96 %/300 MPa/9 min for *C. vulgaris*; water/500 MPa/15 min and ethanol 96 %/420 MPa/9 min for *N. oceanica*; water/420 MPa/9 min and ethanol 96 %/500 MPa/15 min for *C. amblystomatis*; water/420 MPa/9 min and ethanol 96 %/420 MPa/9 min for *P. tricornutum*; and water/300 MPa/9 min and ethanol 96 %/300 MPa/9 min for *T. chui*.

3.4. Cytotoxicity of the extracts

The cytotoxicity and the IC50 (reduction of the metabolic activity by

Table 1
Total phenolic content (μg GAE/mg dry weight) total proteins content (μg BSA/mg dry weight), chlorophyll *a* and total chlorophyll content (μg/mg dry weight) of *N. oceanica*, *C. amblystomatis*, *C. vulgaris*, *T. chui* and *P. tricornutum* extracts obtained under the selected HPE conditions and respective controls. Values are expressed as mean ± SD of three determinations and corrected in accordance to the yield of the extract.

	Extraction pressure/time	Solvent	Total phenolic content	Total proteins	Chlorophyll a	Total Chlorophyll
Chlorella vulgaris	420 MPa/9 min	Water	0.003 ± 0.001	55.180 ± 1.951	363.079 ± 106.237	1047.574 ± 285.850
	300 MPa/9 min	Ethanol 96 %	0.004 ± 0	52.176 ± 1.131	68.596 ± 0.903	92.843 ± 1.066
	Control/9 min	Water	0.002 ± 0	48.203 ± 1.442	260.503 ± 48.63	753.063 ± 130.895
	Control/9 min	Ethanol 96 %	0.007 ± 0.004	27.684 ± 3.566	59.956 ± 1.365	82.769 ± 1.872
Nannochloropsis oceanica	500 MPa/15 min	Water	0.005 ± 0	14.950 ± 1.237	46.172 ± 2.799	124.949 ± 8.803
	420 MPa/9 min	Ethanol 96 %	0.004 ± 0.001	19.822 ± 1.187	887.988 ± 94.147	1087.020 ± 134.320
	Control/15 min	Water	0.001 ± 0	11.694 ± 0.286	15.748 ± 2.918	41.470 ± 8.189
	Control/9 min	Ethanol 96 %	0.003 ± 0.001	13.577 ± 0.137	692.145 ± 8.080	948.778 ± 17.232
Chlorococcum amblystomatis	420 MPa/9 min	Water	0.001 ± 0	5.794 ± 0.983	5.711 ± 0.797	15.985 ± 1.286
	500 MPa/15 min	Ethanol 96 %	0 ± 0	0.448 ± 0.098	7.771 ± 0.188	13.470 ± 0.275
	Control/9 min	Water	0.001 ± 0	3.150 ± 1.053	0.609 ± 0.530	1.798 ± 0.416
	Control/15 min	Ethanol 96 %	0 ± 0	0.284 ± 0	0.054 ± 1.325	0.256 ± 0.094
Phaeodactylum tricormutum	420 Mpa/9 min	Water	0.004 ± 0	24.449 ± 2.435	74.104 ± 3.9161	158.812 ± 7.150
	420 Mpa/9 min	Ethanol 96 %	0.004 ± 0	6.801 ± 0.568	309.011 ± 1.842	414.243 ± 4.208
	Control/9 min	Water	0.001 ± 0	11.472 ± 3.846	39.708 ± 0.530	94.567 ± 2.207
	Control/9 min	Ethanol 96 %	0.003 ± 0	7.257 ± 0.165	345.924 ± 6.409	451.770 ± 10.673
Tetraselmis chui	300 MPa/9 min	Water	0.0017 ± 0.001	11.974 ± 1.628	31.071 ± 5.025	79.190 ± 13.238
	300 MPa/9 min	Ethanol 96 %	0.0062 ± 0	10.006 ± 1.407	887.988 ± 94.105	1087.020 ± 134.320
	Control/9 min	Water	0.0242 ± 0.004	8.746 ± 0.753	15.748 ± 2.918	41.470 ± 8.189
	Control/9 min	Ethanol 96 %	0.0044 ± 0	7.754 ± 0.215	482.990 ± 16.828	623.537 ± 20.454

50 %) of the extracts were evaluated in different skin cells. The IC50 varied with the cell type and extraction conditions (Fig. 3 and Supplementary Fig. S1), except for the *T. chui* extracts, which IC50 was above 500 μg/mL independently of those. In fibroblasts (hDFBs) and adipocytes (hADs), the IC50 of extracts from *N. oceanica*, *C. amblystomatis* and *C. vulgaris* was above 500 μg/mL, whereas from *P. tricornutum* varied between 105 and 10,900 μg/mL depending on the extraction conditions. This dependence was also observed with keratinocytes (hKCs) and melanocytes (hMels), in which the IC50 varied between 31.20 and 291.00 μg/mL and between 30.50 and 280.00 μg/mL, respectively.

Particularly for the selected extracts (Fig. 4), in the presence of the aqueous extracts of *C. vulgaris* the metabolic activity of hDFBs ($p = 0.0006$, at 500 μg/mL; $p = 0.0004$, at 100 μg/mL; $p = 0.0178$, at 10 μg/mL) and hADs ($p = 0.0374$, at 500 μg/mL; $p = 0.0131$, at 100 μg/mL) increased, while it decreased for hMels ($p = 0.0028$, at 500 μg/mL), in relation to control cultures (absence of extracts). As for the ethanolic extract at 500 μg/mL, the metabolic activity of both hKCs ($p = 0.0004$) and hMels ($p < 0.0001$) decreased when cultured in its presence. In respect to *N. oceanica*, the metabolic activity of hDFBs ($p = 0.0131$, at 10 μg/mL) and hMels ($p = 0.0005$ at 500 μg/mL; $p = 0.0328$ at 100 μg/

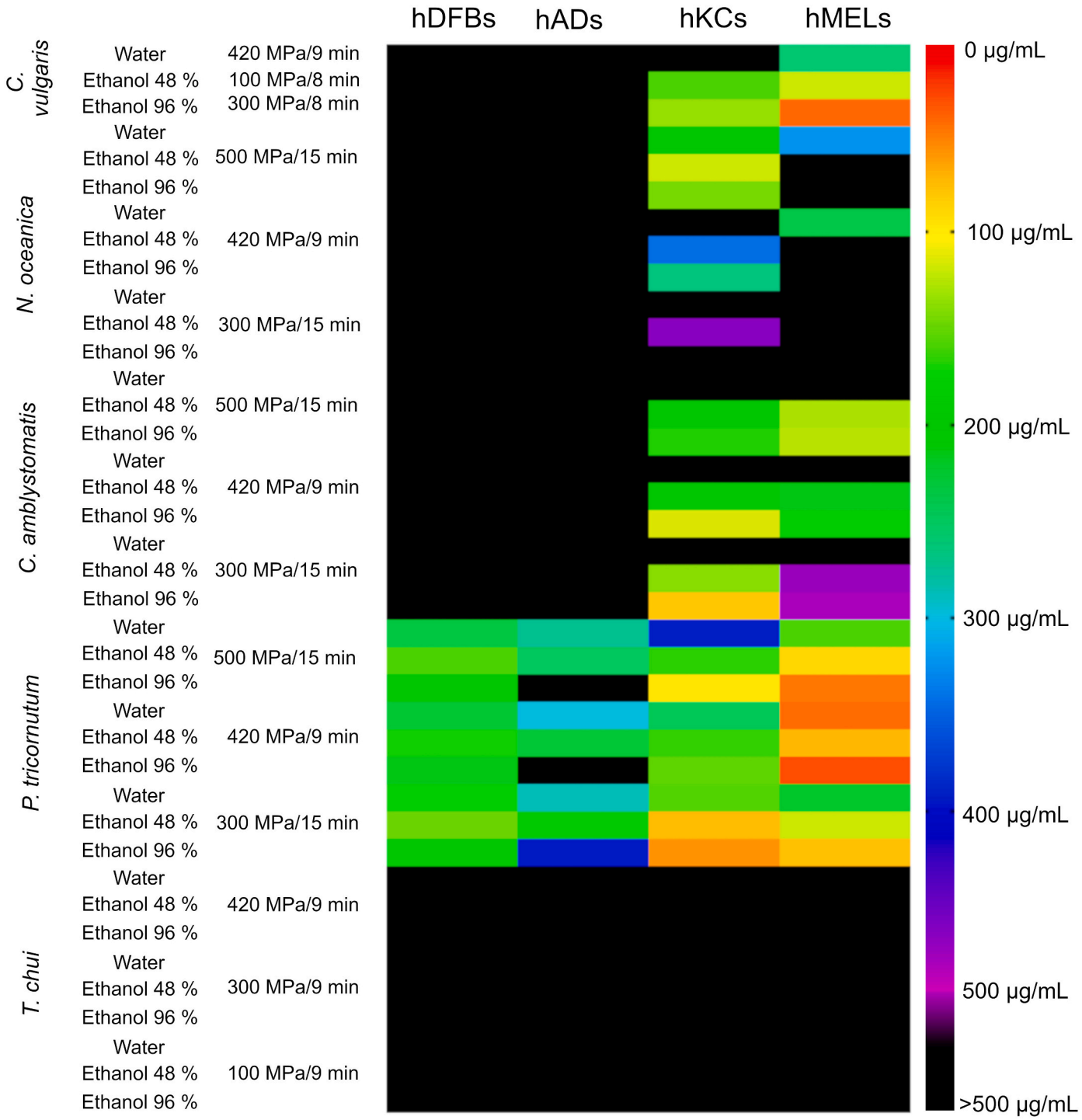

Fig. 3. Heat map representation of the IC50 of *N. oceanica*, *C. amblystomatis*, *C. vulgaris*, *T. chui* and *P. tricornutum* HPE extracts in skin cells. Concentrations between 0 and 500 μg/mL were tested (color bars) and absence of cytotoxicity at the highest concentration is represented by the black bars.

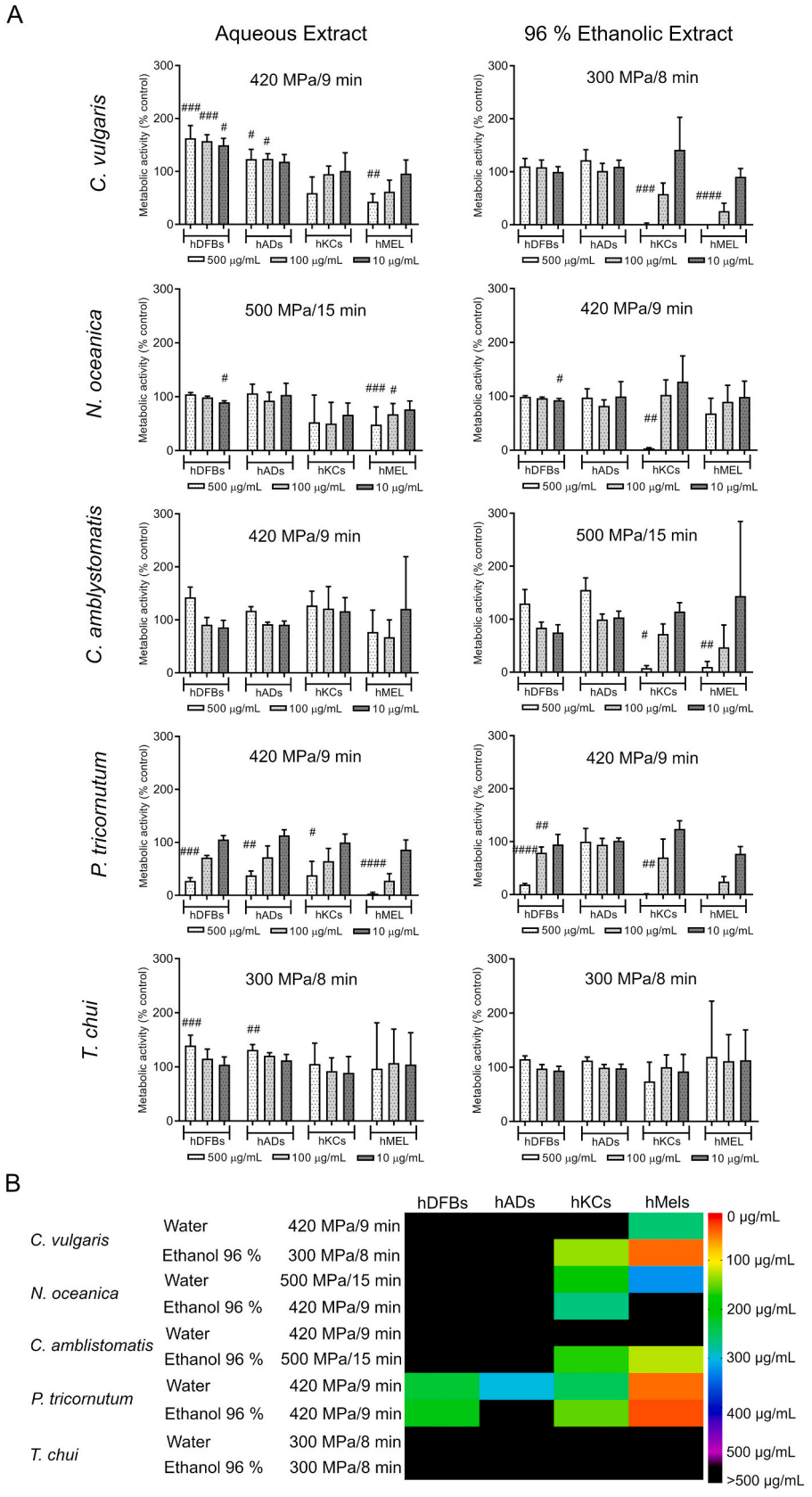

Fig. 4. Cytotoxicity of the *C. vulgaris*, *N. oceanica*, *C. amblystomatis*, *P. tricornutum* and *T. chui* HPE extracts obtained under the selected HPE conditions. A: Quantification of the metabolic activity of skin cells upon incubation with the extracts for 3 days. B: Heat map representation of the IC50 of the extracts. Concentrations between 0 and 500 µg/mL were tested (color bars) and absence of cytotoxicity at the highest concentration is represented by the black bars. # - significantly different to control culture (1 % solvent: water or 96 % ethanol). # - $p < 0.05$, ## - $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$.

mL), and hDFBs ($p = 0.0005$, at 10 μg/mL) and hKCs ($p = 0.0328$, at 500 μg/mL) was reduced in the presence of aqueous and ethanolic extracts, respectively. Regarding *C. amblystomatis*, no significant metabolic activity changes were detected for the aqueous extracts, but the metabolic activity of hKCs ($p = 0.0125$) and hMels ($p = 0.0060$) was reduced in the presence of the ethanolic extracts at 500 μg/mL. A decrease in the metabolic activity of all skin cells was observed in the presence of the *P. tricornutum* aqueous extracts at 500 μg/mL. A similar trend was also evident for the ethanolic extracts at concentrations between 500 and 100 μg/mL, except for hADs which presented a IC50 higher than 500 μg/mL. In respect to *T. chui*, the metabolic activity of hDFBs ($p = 0.0010$) and hADs ($p = 0.0075$) increased when cultured in the presence of aqueous extracts at 500 μg/mL, while no significant metabolic activity changes were detected for the ethanolic extracts.

4. Discussion

Microalgae are an important source of cosmetic ingredients that can be obtained with low environmental impact. A sustainable extraction is part of the process thus, herein HPE using environmentally friendly solvents was explored to obtain extracts of *C. vulgaris*, *N. oceanica*, *T. chui*, *C. amblystomatis*, *P. tricornutum* with cosmeceutical potential. The application of HPE in combination with water as a solvent yielded the most promising results in component extraction. The use of such solvents provides a lower environmental impact, presenting an advantage over conventional methods like chemical treatment with organic solvents [36]. Additionally, HPE breaks the microalgae cell wall and membrane, allowing a faster and efficient solvent permeation than conventional extraction processes. Indeed, HPE method herein used in this study resulted in a higher yield of phenolic compounds in the *P. tricornutum* extract than the previously reported freeze-drying method combined with methanol [37]. Moreover, HPE is advantageous due to its lower time and energy consumption in comparison to conventional methods such as freeze-drying [38]. Since HPE extraction is conducted at room temperature, it is also expected to preserve heat-sensitive compounds [9], offering an advantage over methods like ultrasonication and microwaves, which are performed at higher temperatures causing protein denaturation [36].

We confirmed that higher pressure/time conditions resulted in higher extraction yield, especially compared to control conditions at atmospheric pressure. Yet, the optimal extraction conditions – maximum yield - were dependent on the microalgae species. Previous reports have shown that thin-wall cells required a lower pressure to extract phytochemicals [39,40], therefore, the observed difference could be due to varied cell size and cell wall thickness among species. Like the yield, the composition of the obtained extracts was positively affected by HPE, which is in accordance with previous studies using wet *Haematococus pluvialis* and *Porphyridium cruentum* microalgae slurries [41,42]. Moreover, the type of solvent impacted the components of the extracts which also differed according to the microalgae species. Previous studies also showed that differences in the phytochemical profile of *C. vulgaris* and *P. tricornutum* affected the optimal conditions of pressurized liquid extraction in combination with different solvents [43]. Herein, while the phenolic content was similar for all microalgae extracts obtained with HPE, their protein content was different. This might be because the native protein content of microalgae varies between 6 % to 70 % [1]. In general, the phytochemical results obtained in this work are in accordance with results described in the literature [44–47]. Moreover, the concentrations of both chlorophyll a and total chlorophyll we obtained were superior than those in other works [45,46].

Given that those components of the microalgae extracts are bioactive, an effect on skin cell behavior was confirmed. The selection of various skin cells for this study were based on the potential use of the obtained extracts as ingredients in cosmetic formulations with different activities. Keratinocytes and fibroblasts are the most commonly used cells to evaluate safety of cosmetic ingredients, as well as their efficacy regarding skin hydration, barrier function, rejuvenation and antiaging [48]. In turn, melanocytes are frequently used to evaluate skin hyperpigmentation [48] while adipocytes have been applied for the evaluation of lipolysis [49], particularly relevant in the context of body contouring. Moreover, recent research has highlighted the significant relationship between alterations in skin adipose tissue and aging [50], emphasizing the importance of evaluating the effects of the extracts on these cells. Previous reports showed higher metabolic activity in fibroblasts and keratinocytes cultured in the presence of *C. vulgaris* extracts (50 μg/mL and 1 μg/mL, respectively) [16,51]. In turn, *N. oceanica* extracts at a concentration of 200 μg/mL had a cytotoxic effect in fibroblasts [23]. These results indicate a dependence of the cell response with the concentration of the extract and the species of microalgae. Our results also showed that the cytotoxicity of extracts was dependent on the tested extract concentration within each specie, except for the *T. chui* which IC50 was above the highest concentration tested (500 μg/mL). Directly, cell responses are linked to a specific phytochemical content associated to the extraction method or conditions. The higher metabolic activity or reduced cytotoxicity of *C. vulgaris* and *N. oceanica* extracts was attributed to the protein and pigment contents as well as the phenolic compounds of these microalgae extracts [17,18,23,47]. Hence, the higher content in pigments (chlorophyll a and total chlorophyll) detected in our *C. vulgaris* aqueous and *N. oceanica* and *T. chui* ethanolic extracts and the higher protein content of *Chlorella vulgaris* aqueous extracts might be responsible for the observed positive effects over skin cells.

Interestingly, the IC50 also varied with cell type; keratinocytes and melanocytes presented a higher sensitivity to the extracts than fibroblasts and adipocytes. A higher sensitivity of keratinocytes compared to fibroblasts has been previous reported in extracts from *Planktochlorella nurekis* [52] and areca nut [53]. While the mechanisms involved in this response are mostly unknown, the level of ROS seems to play a critical role. The higher sensitivity of keratinocytes was related to an increased production of ROS in these cells due to the suppression of catalase activity by areca nut [53], *Medicago sativa L.* [54] and *Cornus mas* L. extracts [55]. Moreover, in the fibroblasts, the basal levels of catalase and Heme oxygenase-1 activity, both important in the protection of cells against ROS, were higher than in keratinocytes [53].

In summary, the applied HPE methodology allowed obtaining *Chlorella vulgaris* extracts rich in proteins using water as solvent. Moreover, the use of ethanol permitted to enrich *Nannochloropsis oceanica* and *Tetraselmis chui* extracts in photosynthetic pigments. Proteins are known for their antioxidant and immune-stimulating activities, while also increasing the production of ECM components which are important features for anti-aging cosmetics [1]. Chlorophylls are also considered superior to synthetic dyes and suitable for cosmetics due to its coloring as well as antioxidant, together with phenolic compounds [5], and anti-inflammatory properties [1,56]. It is also linked to wound healing effects, and stimulation of tissue growth and repair [10]. As such, those HPE extracts from *C. vulgaris*, *N. oceanica* and *T. chui* are expected to have an improved capacity as antioxidant while promoting an anti-aging effect due to their increased content in proteins and chlorophylls.

5. Conclusions

In this work we propose a sustainable high-pressure extraction (HPE) process to obtain extracts from *C. vulgaris*, *N. oceanica*, *C. amblystomatis*, *P. tricornutum* and *T. chui* with cosmeceutical potential. HPE in conjugation with different solvents increased the extraction yield and the concentration of phytochemical components, specially at higher pressure/time conditions. While *C. vulgaris* aqueous extracts presented higher protein content, *N. oceanica* and *T. chui* ethanolic extracts presented higher amounts of photosynthetic pigments. The effects of HPE extracts in the metabolic activity of skin cells were also dependent of strain and cell type. In fibroblasts and adipocytes, extracts presented a

higher IC50. In contrasts, the IC50 was below 500 μg/mL for most extracts in keratinocytes and melanocytes. Overall, extracts of sustainably-grown microalgae obtained by a sustainable pressure-based extraction method are a promising source of natural ingredients for cosmeceutical applications.

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CRediT authorship contribution statement

Mónica P. Garcia: Writing – original draft, Methodology, Investigation, Formal analysis. **Ana Regueiras:** Writing – original draft, Methodology, Investigation, Formal analysis. **Graciliana Lopes:** Investigation, Formal analysis. **Gabriela Matos:** Writing – original draft, Methodology, Investigation, Formal analysis. **Lucília P. da Silva:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Mariana T. Cerqueira:** Writing – original draft, Methodology, Investigation, Formal analysis. **Helena Cardoso:** Writing – original draft, Methodology, Investigation, Formal analysis. **Nádia Correia:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Jorge A. Saraiva:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Joana L. Silva:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Rosário Martins:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Alexandra P. Marques:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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