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Unveiling the potential of Pseudococcomyxa simplex: a stepwise extraction for cosmetic applications

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Abstract:	Microalgae are gaining attention as they are considered green fabrics able to synthesize many bioactive metabolites, with unique biological activities. However, their use at an industrial scale is still a challenge because of the high costs related to upstream and downstream processes. Here, a biorefinery approach was proposed, starting from the biomass of the green microalga Pseudococcomyxa simplex for the extraction of two classes of molecules with a potential use in cosmetic industry. Carotenoids were extracted first by an ultrasound assisted extraction, and then, from the residual biomass, lipids were obtained by a conventional extraction. The chemical characterization of the ethanol extract indicated lutein, a biosynthetic derivative of α-carotene, as the most abundant carotenoid. The extract was found to be fully biocompatible on a cell-based model, active as antioxidant and with an in-vitro anti-aging property. In particular, the lutein-enriched fraction was able to activate Nrf2 pathway, which plays a key role also in aging process. Finally, lipids were isolated from the residual biomass and the isolated fatty acids fraction was composed by palmitic and stearic acids. These molecules, fully biocompatible, can find application as emulsifiers and softener agents in cosmetic formulations. The exploration of an untapped microalgal species is a sustainable alternative to conventional formulations.
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Cover Letter

UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II
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DIPARTIMENTO DI SCIENZE CHIMICHE

Naples, March 21, 2024

Head of Applied Microbiology & Biotechnology

Dear Editor,

Please find enclosed the manuscript entitled "Unveiling the potential of *Pseudococcomyxa simplex*: a stepwise extraction for cosmetic applications" by Paola Imbimbo, Enrica Giustino, Alfonso Ferrara, Gerardo Alvarez-Rivera, Hassan Annaz, Elena Ibanez, Maria Chiara Di Meo, Armando Zarrelli, Daria Maria Monti.

In this paper, we exploited microalgae with a multicomponent biorefinery approach by a cascade extraction process. A complete protocol was set up for antioxidants and fatty acids extraction. In particular, carotenoids were extracted first and found to be enriched in lutein. The extract showed *in-vitro* anti-aging property, was fully biocompatible on a cell-based model, and active as antioxidant through the activation of the Nrf2 pathway. Finally, lipids were isolated from the residual biomass and the isolated fatty acids fraction was composed by palmitic and stearic acids, fully biocompatible molecules. To our knowledge, this is the first report on *P. simplex* biorefinery in which the extracted molecules can find application also in cosmetic formulations.

We believe that our paper would be of interest for Applied Microbiology & Biotechnology readers, considering the wide ranged perspectives and the impact that it might have.

Thank you in advance for your consideration,

Sincerely yours,

Paola Imbimbo and Daria Maria Monti
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1 **Unveiling the potential of *Pseudococcomyxa simplex*: a stepwise extraction for cosmetic**
2 **applications**

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18 Keywords: *Pseudococcomyxa simplex*; Carotenoids; Fatty acids; Biorefinery; Cosmetics.

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27 **Abstract**

28 Microalgae are gaining attention as they are considered green fabrics able to synthesize many
29 bioactive metabolites, with unique biological activities. However, their use at an industrial scale is
30 still a challenge because of the high costs related to upstream and downstream processes. Here, a
31 biorefinery approach was proposed, starting from the biomass of the green microalga
32 *Pseudococcomyxa simplex* for the extraction of two classes of molecules with a potential use in
33 cosmetic industry. Carotenoids were extracted first by an ultrasound assisted extraction, and then,
34 from the residual biomass, lipids were obtained by a conventional extraction. The chemical
35 characterization of the ethanol extract indicated lutein, a biosynthetic derivative of α-carotene, as the
36 most abundant carotenoid. The extract was found to be fully biocompatible on a cell-based model,
37 active as antioxidant and with an *in-vitro* anti-aging property. In particular, the lutein-enriched
38 fraction was able to activate Nrf2 pathway, which plays a key role also in aging process. Finally,
39 lipids were isolated from the residual biomass and the isolated fatty acids fraction was composed by
40 palmitic and stearic acids. These molecules, fully biocompatible, can find application as emulsifiers
41 and softener agents in cosmetic formulations. The exploration of an untapped microalgal species is a
42 sustainable alternative to conventional formulations.

43 **Key Points**

44 - *Pseudococcomyxa simplex* has been explored in a cascade approach.
45 - Lutein is the main extracted carotenoid and has antioxidant and anti-aging activity.
46 - Fatty acids are mainly composed of palmitic and stearic acids.

47 **Introduction**

48 Cosmetic is one of the most remunerative industrial sectors in the world, constantly growing since 49 2004. It has been estimated that, in 2028, the revenues of the global cosmetic market will be nearly 50 129 billion USD. In particular, the beauty market is dominated by skincare, which is expected to reach 51 221.38 billion UDS by 2030 with a CAGR of 4.79% (https://straitsresearch.com/report/facial- 52 skincare-products-market, accessed on 14 March 2024). Nowadays, consumers prefer to use natural 53 cosmetics that are produced from natural sources that can be considered safe for humans and 54 environment. This trend inversion is due not only to the growing environmental consciousness of the 55 consumers, but also to their awareness of health issues that can occur by using synthetic compounds, 56 such as hyperactivity, allergic reactions, or other side effects (Tang et al. 2020). Thus, different 57 cosmetic companies are using biotechnology to ensure quality, efficacy, and safety of the natural 58 products (Bouzroud et al. 2023). Among natural sources, microalgae have gained attention as they 59 are considered green fabrics able to synthesize a plethora of bioactive metabolites, endowed with 60 unique biological activities (Liberti et al. 2023). Moreover, they are perceived as vegan, natural and 61 healthy by consumers. To date, only a few strains have been exploited for cosmetic purposes, such as 62 *Spirulina*, *Dunaliella*, *Chlorella,* and *Haematococcus* (Yarkent et al. 2020). Despite the considerable 63 potential of microalgae, some issues still limit their full industrialization, such as high upstream and 64 downstream costs (Imbimbo et al. 2020). To make the use of microalgae sustainable and feasible, the 65 biorefinery approach has been proposed as the technology is able to implement the circular economy 66 and lowers the overall process costs (Igbokwe et al. 2022).

67 Carotenoids are pigments which can be used in different fields, such as the food and pharmaceutical 68 industry, and recently, due to the potent antioxidant activity, they have attracted interest to be used as 69 active ingredients in cosmetic formulations. Currently, the market is dominated by astaxanthin, β- 70 carotene, zeaxanthin, lutein, and lycopene (Sathasivam and Ki 2018),

71 Lipids, and more in general oils, are one of the major components in cosmetic cream formulations
72 (Franco et al. 2022). Based on their nature, they can be used for different purposes, such as softener
73 agents, emulsifiers, detergents and skin lighteners (De Luca et al. 2021). Fatty acids are used in
74 cosmetics as emollients to improve the skin-hydration, and as emulsifiers, since they can act as
75 thickening agents. It has been reported that the ideal length of the carbon chain for fatty acids-based
76 emulsion is 16-18, which correspond to palmitic and stearic acid (Cochran and Anthonavage 2015).
77 Fatty acids are also physiological skin components that ensure the maintenance of skin barrier
78 functions (Knox and O'Boyle 2021). In this paper we proposed a biorefinery approach starting from
79 the biomass of the green microalga *Pseudococcomyxa simplex* for the extraction of two classes of
80 molecules with a potential use in cosmetic industry. Carotenoids were extracted as the first class of
81 molecules by an ultrasound assisted extraction coupled to maceration, and then, from the residual
82 biomass, lipids were obtained by a conventional extraction. Finally, a chemical and biological
83 characterization of both classes of molecules have been carried out to investigate a possible use of
84 these molecules in cosmetic field.

85 **Materials and Methods**

86 **Reagents**

87 All the reagents, unless differently specified, were purchased from Sigma-Aldrich (Milan, Italy).

88 **Microalgae strain and cultivation**

89 *Pseudococcomyxa simplex* (ACUF 127) was provided by the Algal Collection of the University
90 Federico II (ACUF, www.acuf.net). The cultivation was carried out in bubble column
91 photobioreactors in Bold Basal Medium (BBM) at 24 ± 2 °C with a constant light intensity of 100
92 PARs [($\mu mol_{photons}/m^2$)/s]. The culture was mixed by bubbling air through a sintered glass tube placed
93 at the bottom of each reactor. Algal growth was monitored by measuring the absorbance at 730 nm.
94 The dry weight determination was carried out *via* conversion between the Optical Density (O.D.) and
95 the biomass dry weight at the end of the exponential growth phase. The conversion factor was: 1 O.D.
96 corresponded to 0.22 mg dry weight. The biomass concentration achieved at the end of the
97 exponential growth phase was 0.7 $g_{D.W.}$/L.

98 **Pigments extraction and characterization**

99 Pigments were extracted using ethanol as solvent, as previously reported (Imbimbo et al. 2023).
100 Briefly, 200 mg of dry weight (D.W.) of alga were suspended in 4 mL of pure ethanol and disrupted
101 by ultrasonication (40% amplitude, 4 min on ice, Bandelin SONOPULS HD 3200, tip MS73). The
102 volume was adjusted to 20 mL and the mixture was shaken for 24 h at 250 rpm at 4 °C in the dark.
103 Pigments were recovered in the supernatants by centrifugation at 5000 *g* for 10 min and then ethanol
104 was removed under N_2 stream. The extraction yield was determined gravimetrically.

105 Carotenoids and pigments identification was performed by HPLC-DAD-APCI-QTOF-MS/MS. The
106 analysis of the extracts was carried out in an Agilent 1290 UHPLC system (Ultrahigh Performance
107 Liquid Chromatography) equipped with a diode-array detector (DAD), coupled to an Agilent 6540
108 quadrupole-time-of-flight mass spectrometer (q-TOF MS) equipped with an atmospheric pressure

109 chemical ionization (APCI) source. A Thermo Fisher Scientific Accucore C30 column (2.6 µm, 4.6 x
110 50 mm) was used at 30 ºC. Separation was achieved using a 12 min gradient program from 100%
111 mobile phase A (90% Methanol, 7% MTBE, 3% water) and 0% mobile phase B (90% MTBE, 10%
112 Methanol) to 0% mobile phase A and 100% mobile phase B, and kept constant for 1.5 min before
113 returning to the initial conditions within 1.5 min. The total run time was 15 min at a flow rate of 0.8
114 mL/min. The mass spectrometer was operated in positive ionization mode (APCI+), with gas
115 temperature at 300 ºC; drying gas at 8 L/min; vaporizer temperature at 350 ºC; nebulizer pressure at
116 40 Psi; capillary voltage at 3500 V; corona+: 4 µA; fragmentor voltage at 110 V and skimmer voltage
117 at 45 V. The MS and auto MS/MS modes were set to acquire *m/z* values ranging between 25-1500, at
118 a scan rate of 10 spectra per second. Auto MS/MS mode was operated at two collision-induced
119 dissociation energies: 20 and 40 eV and selecting 4 precursor ions per cycle at a threshold of 200
120 counts.

121 **Lipids extraction and characterization**

122 Lipids were recovered by using the original Bligh and Dyer protocol (Bligh and Dyer 1959).
123 Extractions were carried out on 300 mg of both freeze-dried biomass and residual freeze-dried
124 biomass (i.e., after pigment extraction) using chloroform, methanol, and water in a ratio 2:2:1 (v/v/v)
125 for 1 h at room temperature. At the end of the extraction, the hydrophobic phase was recovered by
126 centrifugation and the extract was dried under N_2 stream. The lipid extract was then fractionated in
127 three different lipid classes (i.e. neutral lipids, fatty acids, and phospholipids) by performing a solid
128 phase extraction (SPE) as previously described (Imbimbo et al. 2019). The recovered fatty acids were
129 first derivatized by transforming them into the corresponding methyl esters and then identified and
130 quantified by GC−MS analysis (Crescenzo et al., 2015).

131 **Biological characterization**

132 ***In vitro anti-aging assays***

133 The *in vitro* anti-aging activity was evaluated by anti-tyrosinase and anti-elastase assays, as described
134 by Mahdi et al. (Mahdi et al. 2024). Kojic acid was used as commercial inhibitor.

135 ***Cell culture and biocompatibility of ethanol extract***

136 Immortalized human keratinocytes (HaCaT) were from Innoprot (Biscay, Spain) and immortalized
137 murine fibroblasts (BALB/c-3T3) were from ATCC (VA, USA). Cells were cultured in Dulbecco's
138 modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum (HyClone), 2 mM
139 *L*-glutamine, and antibiotics, under a 5% CO_2 humidified atmosphere at 37 °C. The biocompatibility
140 of either ethanol extract or FAs was tested. HaCaT cells were seeded in 96-well plate at a density of
141 2.5×10^3 cells per well, whereas BALB/c-3T3 at a density of 3×10^3 cells per well. HaCaT cells were
142 incubated with increasing concentration (from 0.5 to 100 µg/mL) of both ethanol extract and FAs for
143 24 and 48 h, whereas BALB/c-3T3 only with ethanol extract. At the end of the incubation, cell
144 viability was assessed by the MTT assay, as previously reported (Liberti et al. 2023). Cell viability
145 was expressed as the percentage of viable cells in the presence of the extract compared to the controls,
146 represented by untreated cells and cells supplemented with identical volumes of DMSO.

147 ***Sodium arsenite stress induction and biochemical analyses***

148 HaCaT cells were pre-treated with 90 µg/mL of ethanol extract for 2 h. Then, cells were stressed with
149 300 µM sodium arsenite ($NaAsO_2$) for 1 h as previously described (Sobeh et al. 2019). Immediately
150 after $NaAsO_2$ stress induction, intracellular ROS levels were determined by DCFDA assay, as
151 previously reported (Imbimbo et al. 2023), whereas intracellular glutathione (GSH) levels were
152 measured by performing 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay, as reported by (Laezza
153 et al. 2024).

154 ***Western blot analysis***

155 90 min after stress induction, HaCaT cells were detached by trypsin and lysate in lysis buffer (0.1 M
156 Tris HCl pH 7.4, 0.3 M NaCl and 0.5% NP40), supplemented with protease and phosphatase

157 inhibitors. After 30 min incubation on ice, lysates were centrifuged at 14,000 *g* for 30 min at 4 °C.
158 Supernatants were collected and protein concentration was determined by the Bradford assay. 80 µg
159 of proteins were separated by SDS-PAGE and analyzed by Western blotting using specific antibodies:
160 anti-phospho-p38, anti-phospho-MAPKAPK-2 from Cell Signalling (Danvers, MA, USA); anti-Nrf2
161 from Bioss Antibodies (Woburn, MA, USA); anti-HO-1 from Bethyl laboratories INC.
162 (Montgomery, TX, USA), anti-B-23 and anti-β-actin. Band detection and densitometric analyses
163 were performed using a ChemiDoc (Biorad, Hercules, CA, USA), according to the manufacturer's
164 instruction.

165 **Statistical analyses**

166 Samples were tested in three independent analyses, each carried out in triplicate. Results are presented
167 as mean of results obtained after three independent experiments (mean ± SD) and compared by one-
168 way ANOVA according to the Bonferroni's method (post hoc) using Graphpad Prism for Windows,
169 version 6.01.

170 **Results**

171 **Pigments extraction and characterization**

172 The proposed biorefinery strategy consists in the extraction of two different classes of molecules,
173 pigments and lipids, starting from *P. simplex* biomass. The order of the extraction was chosen by
174 following two different criteria: the polarity of the target molecules, to ensure that the extraction
175 solvent would not affect the quality of the residual biomass, and the market value of the molecules to
176 be extracted.

177 To obtain pigments, the biomass was harvested, freeze-dried, and treated as described in Materials
178 and Methods section. At the end of the extraction, the mixture was centrifuged, the supernatant dried
179 under N_2 stream, and cells debris were dried and stored for further extraction. The yield of the ethanol
180 extract was 26 ± 3%. To investigate the composition of the ethanol extract, a profiling analysis using
181 a chromatographic HPLC-DAD system hyphenated to a QTOF-MS analyser was used to obtain
182 complementary structural information. UV-vis profiles and HRMS/MS data acquired in positive
183 ionization mode (APCI+), were jointly analysed to increase structural elucidation capacity.

184 The UHPLC-DAD chromatographic profile, shown in **Fig. 1**, revealed the presence of 7 major
185 carotenoids and 6 chlorophylls/chlorophyll derivatives, tentatively identified according to their
186 maximum absorption wavelength (λ_{max}), molecular ion (*m/z*), and main MS/MS fragments obtained
187 by LC-APCI(+)-MS/MS analysis. Structural information of the annotated pigments is summarized in
188 Table S1. From their calculated molecular formulae, chromatographic peaks 1 to 5 were annotated as
189 compounds belonging to the xanthophylls (oxygen-containing carotenoids), whereas compounds 12
190 and 13 were classified as carotenes (hydrocarbon carotenoids). The nitrogen-containing pigments
191 (peaks 6 to 11) were classified as chlorophylls and chlorophyll derivatives. Concentration values were
192 estimated for the identified carotenoids following a semi-quantitative approach using β-carotene as
193 reference standard (**Table 1**).

194 Carotenoids 1-5, 12 and 13 showed three typical maximum absorption wavelengths ranging from 400
195 to 475 nm in their UV-vis spectra. Compounds 1 and 2 are two major carotenoids in *P. simplex*, that
196 coelute under the same peak and show molecular ions at *m/z* 567.4196 ($C_{40}H_{54}O_2$) and *m/z* 601.4251
197 ($C_{40}H_{56}O_4$), respectively. Compound 3 with *m/z* 585.4302 ($C_{40}H_{56}O_3$) shares similar UV-vis
198 absorption profile to compound 2. The first compound was annotated as a didehydro-carotenediol
199 (e.g., diatoxanthin/monadoxanthin), whereas the second and the third were annotated as neoxanthin
200 and the third one as mutatoxanthin-type, respectively; two biosynthetically related epoxicarotenoids
201 and β-carotene derivatives. Peak 4 (*m/z* 569.4353, $C_{40}H_{56}O_2$) was unambiguously annotated as lutein,
202 the most abundant carotenoid identified in the ethanol extract of *P. simplex*, whereas peak 5 (*m/z*
203 551.4247, $C_{40}H_{54}O$) was tentatively identified as crocoxanthin. Both compounds 4 and 5 are
204 biosynthetic derivatives of α-carotene. Additionally, two non-oxygenated carotenoid isomers (peaks
205 12 and 13) were identified as α- and β-carotene (*m/z* 537.4455, $C_{40}H_{56}$) and exhibited a higher
206 retention time, in agreement with their higher lipophilicity. Chlorophylls and their derivatives exhibit
207 two major absorption bands in the visible range, corresponding to the cyclic tetrapyrrole (porphyrin)
208 skeleton, at around 420-460 nm and above 650 nm. Due to operational restrictions, only the first band
209 could be measured in this work. In agreement with λ_{max} values in literature (Almela et al. 2000),
210 compounds 6-7 and 8-9 were annotated as chlorophyll *b* and chlorophyll *a* isomers, corresponding to
211 molecular ions at *m/z* 907.5218 ($C_{55}H_{70}MgN_4O_6$) and *m/z* 893.5426 ($C_{55}H_{72}MgN_4O_5$), respectively.
212 Two additional chlorophyll derivatives, lacking the central Mg-atom, were annotated as pheophytin
213 *a* isomers (compounds 10-11). These demetallized forms are less polar than the corresponding
214 chlorophylls, showing higher retention time in reverse phase columns. The most abundant fragment
215 ions in MS/MS spectra of chlorophyll and its derivatives usually correspond to the fragmentation
216 with the loss of the phytyl chain $[M-278]^+$.

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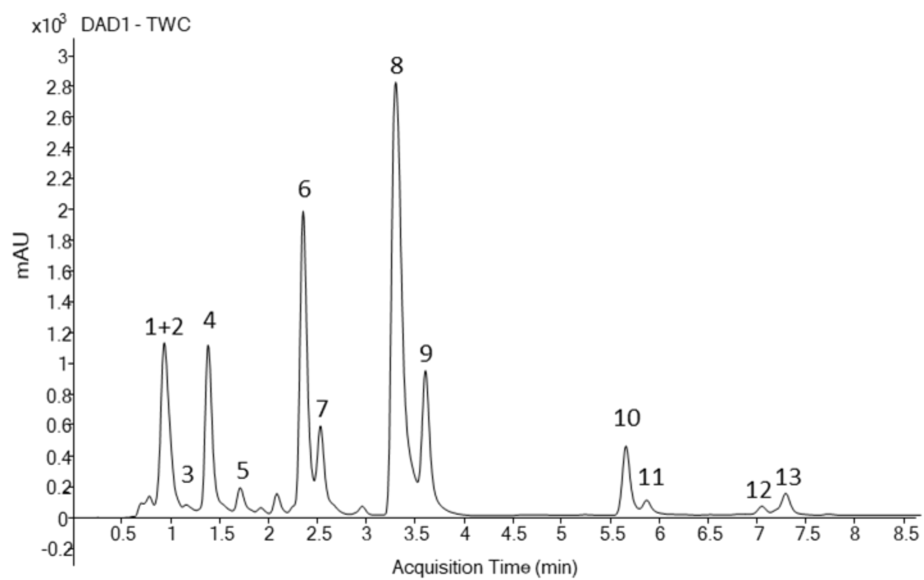

228 **Fig. 1** Representative LC-DAD chromatogram of pigments extracted from *P. simplex*. The TWC (total
229 wavelength chromatogram), in the range 190 to 640 nm, is reported. Peaks identification is reported
230 in **Table S1**

231 **Table 1** Concentration values (mg/$g_{extract}$) of carotenoids identified in the ethanol extracts of *P.*
232 *simplex*

Peak Nº	Compound	Concentration (mg/$g_{extract}$)
1+2	Diatoxanthin/Monadoxanthin/ Neoxanthin	30 ± 1
3	Mutatoxanthin-type	2.5 ± 0.2
4	Lutein	30 ± 1
5	Crocoxanthin	6 ± 1
12	α-Carotene	1.6 ± 0.3
13	β-Carotene	4.6 ± 0.1

233

234 **Lipid extraction and characterization**

235 Lipids were extracted as the second class of molecules by an organic-solvent extraction. The
236 extraction was carried out on the residual biomass (i.e. biomass recovered after pigment extraction)
237 after a drying step. In a parallel experiment, lipids were extracted also from the raw biomass, as
238 benchmark, to verify if the lipids extraction could be affected by the previous pigments extraction.
239 As shown in **Fig. 2a**, the yield of the hydrophobic fraction obtained from the residual biomass was 7
240 ± 1% (grey bar). This value represents a 3-fold decrease compared to the extraction yield of the raw
241 biomass (black bar, 24 ± 3%), thus suggesting that lipids extraction was significantly affected from
242 the pigments extraction.

243 To understand the composition of the isolated lipids, a Solid Phase Extraction (SPE) was carried out
244 to isolate the three lipid classes: neutral lipids, fatty acids, and phospholipids. As shown in **Fig. 2b**,
245 no significant alteration in neutral lipids was observed, whereas phospholipids significantly decreased
246 (from 28% to 13%). However, it is interesting to notice a significant increase in relative fatty acid

247 content in the extract obtained from the residual biomass (24 ± 1%) in comparison with the one

248 obtained from the raw one (7 ± 2%).

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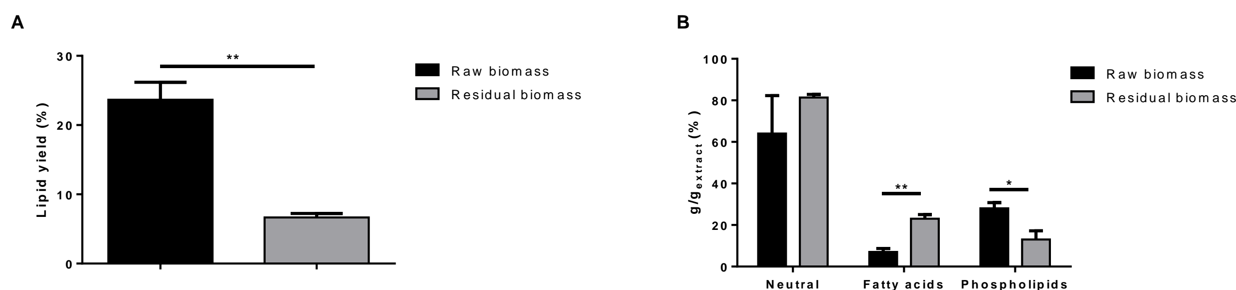

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251 **Fig. 2 Lipids extraction and fractionation. a:** Yields of lipids extracted from *P. simplex* and reported

252 as % with respect to dry weight biomass; **b:** Yield of neutral lipids, fatty acids and phospholipids

253 obtained upon SPE and reported as % with respect of dry weight extract. Black bars refer to raw

254 biomass, gray bars refer to the residual biomass after pigments extraction. Results are reported as

255 means ± SD of at least three independent experiments. * Indicates $p < 0.05$; ** indicates $p < 0.001$.

256 The lines above the bars indicate the samples compared for statistical analysis

257

258 Finally, a gas-chromatography analysis was performed on fatty acids fraction obtained from raw and

259 residual biomass. The results, reported in **Table 2,** suggest that *P. simplex* biomass is enriched in

260 saturated fatty acids (SFA), particularly palmitic and stearic acids. Notably, when fatty acids were

261 recovered from the residual biomass, the total SFA content increased, to such an extent that the extract

262 appears to be composed only of palmitic and stearic acids.

263 **Table 2** Fatty acids composition by Gas chromatography analysis on samples obtained from Raw and
264 Residual biomass after pigment extraction. Saturated, monounsaturated, and polyunsaturated fatty
265 acids are reported as relative percentages

Fatty Acids		Raw biomass (%)	Residual biomass(%)
SFA			
14:0	Myristic	0.56 ± 0.08	0.4 ± 0.1
16:0	Palmitic	30.0 ± 0.8	62 ± 2
18:0	Stearic	15 ± 1	37 ± 2
24:0	Lignoceric	0.13 ± 0.04	0.02 ± 0.01
MUFA			
16:1n7t	*Trans* Palmitoleic	0.04 ± 0.01	0.09 ± 0.01
16:1n7	Palmitoleic	0.20 ± 0.02	0.05 ± 0.04
18:1n9t	*Trans* Oleic	0.13 ± 0.05	0.04 ± 0.01
18:1n9	Oleic	12 ± 1	0.30 ± 0.08
20:ln9	Eicosenoic	0.09 ± 0.01	0.11 ± 0.03
24:ln9	Nervonic	0.03 ± 0.03	0.010 ± 0.001
PUFA			
18:2n6t	*Trans* Linoleic	0.21 ± 0.08	0.06 ± 0.04
18:2n6	Linoleic	20 ± 1	0.24 ± 0.06
18:3n6	γ-Linoleic	21.0 ± 0.8	0.10 ± 0.03
18:3n3	α-Linoleic	0.06 ± 0.01	0.07 ± 0.04
20:2n6	Eicosadienoic	0.73 ± 0.17	0.03 ± 0.03
20:3n6	Dihomo γ-Linoleic	0.03 ± 0.01	0.20 ± 0.02
20:4n6	Arachidonic (AA)	0.06 ± 0.03	0.02 ± 0.01
20:5n3	Eicosapentaenoic (EPA)	0.02 ± 0.01	0.04 ± 0.02
22:4n6	Docosatetraenoic	0.06 ± 0.04	0.010 ± 0.001
22:5n6	Docosapentaenoic-n6	0.05 ± 0.04	0.01 ± 0.01
22:5n3	Docosapentaenoic-n3	N.D.	0.01 ± 0.01
22:6n3	Docosahexaenoic (DHA)	N.D.	0.010 ± 0.001

266

267 **Biological characterization**

268 ***In vitro anti-aging activity***

269 Skin aging is a complex mechanism which depends on endogenous and exogenous factors, such as
270 physiological aging and the continuous exposure to stress factors (Gu et al. 2020). Thus, there is a
271 direct link between aging and oxidative stress. It is well-known that carotenoids can exert a potent
272 antioxidant activity, thus protecting skin cells from stress agents and improving the skin appearance
273 (Mussagy et al. 2023). Different enzymes are involved in skin aging, such as tyrosinase and elastase.
274 The first is involved in the early steps of melanogenesis, whereas the second is involved in elastin
275 degradation, a protein that plays a key role in the maintenance of the elasticity of the overall skin tone
276 (Panwar et al. 2020). Melanin overproduction can cause different dermatological problems, such as
277 hyperpigmentation, which can finally lead to melanoma (Bayrakçeken Güven et al. 2023). The
278 inhibition of these two enzymes can be a way to slow down skin aging, so that a possible inhibitor
279 effect of *P. simplex* carotenoids extract was evaluated *in vitro*. The carotenoids extract was able to
280 inhibit both enzymes, but with a lower extent with respect to kojic acid, a commercial inhibitor. The
281 amount of extract needed to inhibit 50% of the enzyme activity (IC_{50}) was calculated and reported in
282 **Table 3**.

283 **Table 3** Anti-tyrosinase and anti-elastase activity of *P. simplex* carotenoids extract. Values are
284 reported as IC_{50} (mg/mL). Data shown are means ± S.D. of three independent experiments

285

Assay	**Kojic acid IC_{50} (mg/mL)**	***P. simplex* extract IC_{50} (mg/mL)**
Anti-tyrosinase	0.072 ± 0.003	0.48 ± 0.05
Anti-elastase	0.116 ± 0.005	0.55 ± 0.04

286

287 *Effect of carotenoid extract on cell viability*

288 The biocompatibility of the carotenoid extract was evaluated on immortalized human keratinocytes
289 (HaCaT), and on immortalized murine fibroblasts (BALB/c-3T3) by dose- and time-dependent test.
290 Cell viability was assessed by the tetrazolium salt colorimetric (MTT) assay, and cell viability was
291 expressed as the percentage of viable cells in the presence of the extract compared to that of control
292 samples. As shown in **Fig. 3**, extract was fully biocompatible on both HaCaT (**Figure 3a**) and
293 BALB/c-3T3 cells (**Fig. 3b**), as no reduction in cell viability was observed at any of the experimental
294 conditions tested.

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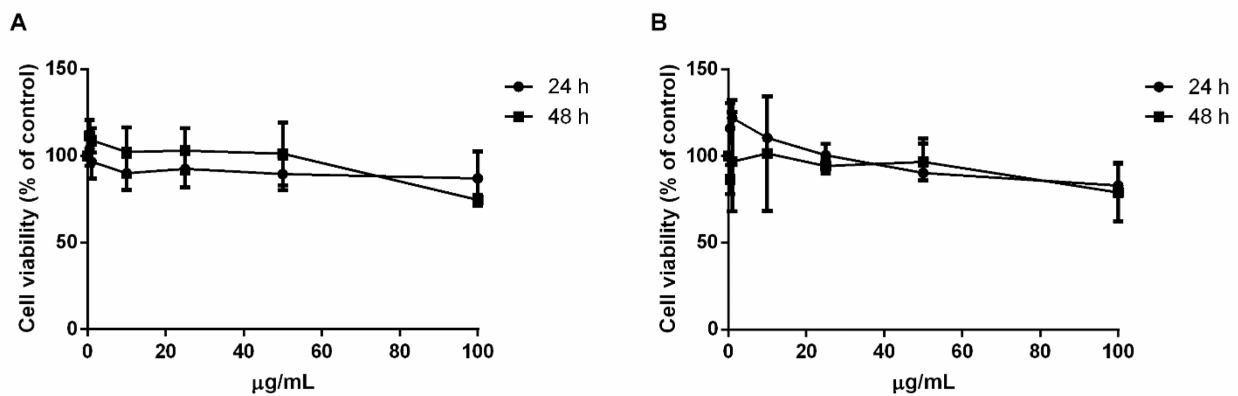


296

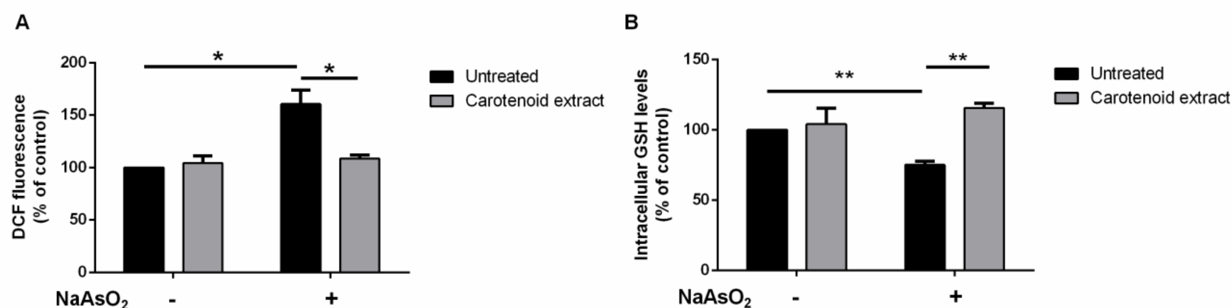
297 **Fig. 3 Cell viability of carotenoids extract on eukaryotic cells.** HaCaT cells (**A**) and BALB/c-3T3
298 (**B**) incubated for 24 h (black circles) or 48 h (black squares) with increasing concentrations (0.5-100
299 μg/mL) of the extract. Cell viability is expressed as a percentage of viable cells in the presence of
300 carotenoids with respect to control cells grown in the absence of the extract. Data shown are means
301 ± S.D. of three independent experiments

302

303 *Protective effect of carotenoid extract against sodium arsenite-induced oxidative stress*

304 It is known that carotenoids are excellent antioxidants. Thus, their protective effect was tested on a
305 cell-based system, in which cells were stressed with sodium arsenite. Organic and inorganic arsenic,

306 are the most common contaminants in air and water (Sobeh et al. 2019). In its trivalent state, arsenic
307 can react with thiol groups in proteins, thus inhibiting their activity. Humans are constantly exposed
308 to this contaminant *via* ingestion, inhalation, and also skin absorption (Ozturk et al. 2022), with severe
309 health problems, such as cancer, cardiovascular disease, diabetes and skin diseases (Rahaman et al.
310 2021). All these conditions are triggered by oxidative stress (Sharifi-Rad et al. 2020). Thus, HaCaT
311 cell were treated as described in Materials and Methods section, and immediately after $NaAsO_2$-stress
312 induction, intracellular ROS levels were measured by using the fluorescent probe 2',7'-
313 Dichlorofluorescin diacetate (H_2-DCFDA). As shown in **Fig. 4a**, in the absence of oxidative stress,
314 no alteration in ROS production was observed when cells were treated with the carotenoid extract,
315 whereas a significant increase in intracellular ROS levels was observed when cells were stressed with
316 $NaAsO_2$. Interestingly, when cells were pre-incubated with ethanol extract prior to stress exposure,
317 an inhibition in ROS production was observed. The protective effect against oxidative stress was
318 confirmed by analyzing the intracellular glutathione levels, a molecule which is normally oxidized
319 during oxidative stress. The intracellular GSH levels were assessed using the 5,5'-Dithiobis-2-
320 nitrobenzoic acid (DTNB) assay. As shown in **Fig. 4b**, the exposure of the cells to $NaAsO_2$ resulted
321 in a significant GSH depletion. Nevertheless, the pre-treatment with carotenoids resulted in the
322 inhibition of GSH depletion, thus confirming the protective effect of the extract against $NaAsO_2$-
323 induced oxidative stress.



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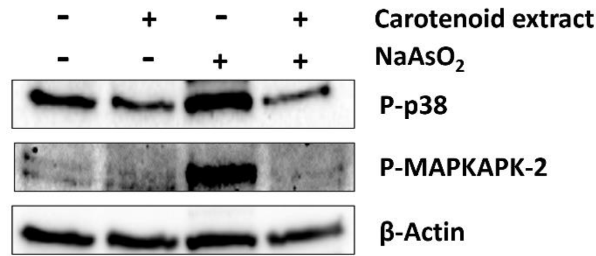
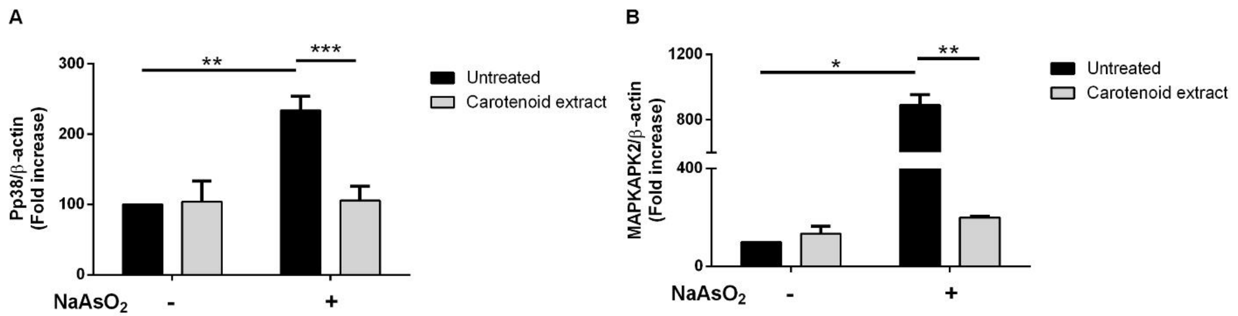
325

326 **Fig. 4 Effect of carotenoid extract on stressed HaCaT cells.** Cells were incubated with 90 µg/mL
327 of *P. simplex* carotenoid extract for 2 h in the absence (-) or in the presence (+) of oxidative stress
328 induced by incubating cells with 300 µM $NaAsO_2$ for 1 h. **a**: intracellular ROS levels measured by
329 DCFDA assay; **b**: intracellular GSH levels measured by DTNB assay. Values are expressed as fold
330 increase with respect to untreated cells. Data shown are means ± S.D. of three independent
331 experiments. * Indicates $p < 0.05$; ** indicates $p < 0.001$. Lines above the bars indicate the samples
332 compared for statistical analysis
333
334 ***Activation of mitogen-activated protein kinases (MAPK) mediated by carotenoid extract***
335 Oxidative stress usually results in the activation of MAP (mitogen-activated protein) kinases pathway.
336 Following oxidative stress insult, p38 is phosphorylated and this causes, in turn, the phosphorylation
337 of its direct target, MAPKAPK-2. Thus, Western blotting analyses were performed to evaluate the
338 protective effect of carotenoids extracted from *P. simplex* biomass. As shown in **Fig. 5**, the carotenoid
339 extract was able to inhibit the phosphorylation of both p38 and MAPKAPK-2. A complete inhibition
340 in the phosphorylation of p38 and MAPKAPK-2 levels was observed when cells were pre-treated
341 with the extract prior to be stressed.
342





343

344 **Fig. 5 Effect of carotenoid extract on the activation of mitogen-activated protein kinase (MAPK)**
345 **cascade upon $NaAsO_2$ stress induction.** HaCaT cells were treated as described in Materials and
346 Methods section. Phosphorylation levels of p38 and MAPKAPK-2 were analyzed by Western
347 blotting. β-actin was used as an internal standard. The relative densitometric analysis of P-p38 (A)
348 and P-MAPKAPK-2 (B) levels is reported. Black bars refer to control cells in the absence (-) or in
349 the presence (+) of sodium arsenite; light grey bars refer to cells incubated with 90 μg/mL of
350 carotenoids. Data shown are the means ± S.D. of three independent experiments. * Indicates $p < 0.05$,
351 ** indicates $p < 0.01$, *** indicates $p < 0.001$. Lines above the bars indicate the samples compared
352 for statistical analysis

353 ***Protection against oxidative stress via the activation of Nrf2 pathway***

354 The nuclear factor E2-related factor 2 (Nrf2) plays a pivotal role in cellular responses to oxidative
355 stress (Li and Kong 2009), as it regulates the expression of antioxidant enzymes and maintains the
356 redox homeostasis (Mansouri et al. 2022). Under physiological conditions, Nrf2 is in the cytosol,
357 associated to the kelch-like ECH-associated protein 1 (KEAP1). In the presence of external stimuli,
358 such as oxidative stress o low exposure to antioxidants, Nrf2 dissociates from KEAP1 and rapidly

359 translocates into the nucleus where it binds the antioxidant responsive elements (ARE) sequences,
360 thus initiating the transcription of over 200 genes involved in the antioxidant response, among which
361 heme oxygenase 1 (HO-1) (Mallard et al. 2020). The activation of Nrf2 pathway is involved in the
362 modulation of skin pigmentation, in wound healing and in the overall protection of the skin from the
363 environmental stresses, so that the use of Nrf2 modulators is considered a useful tool in dermo-
364 cosmetic applications. For this reason, the effect of carotenoid extract in the activation of Nrf2
365 pathway was analyzed. HaCaT cells were treated with 90 µg/mL of carotenoid extract for 10 or 20
366 min and then lysates were analyzed by Western blot analysis, using Nrf2 antibody. As shown in **Fig.
367 6a**, a significant increase in nuclear Nrf2 levels was observed upon 10 min incubation. To corroborate
368 this result, HO-1 levels were also analyzed by Western blot, upon 20- and 30-min incubation. As
369 shown in **Fig. 6b**, a significant increase in HO-1 levels was observed after 20 min incubation.

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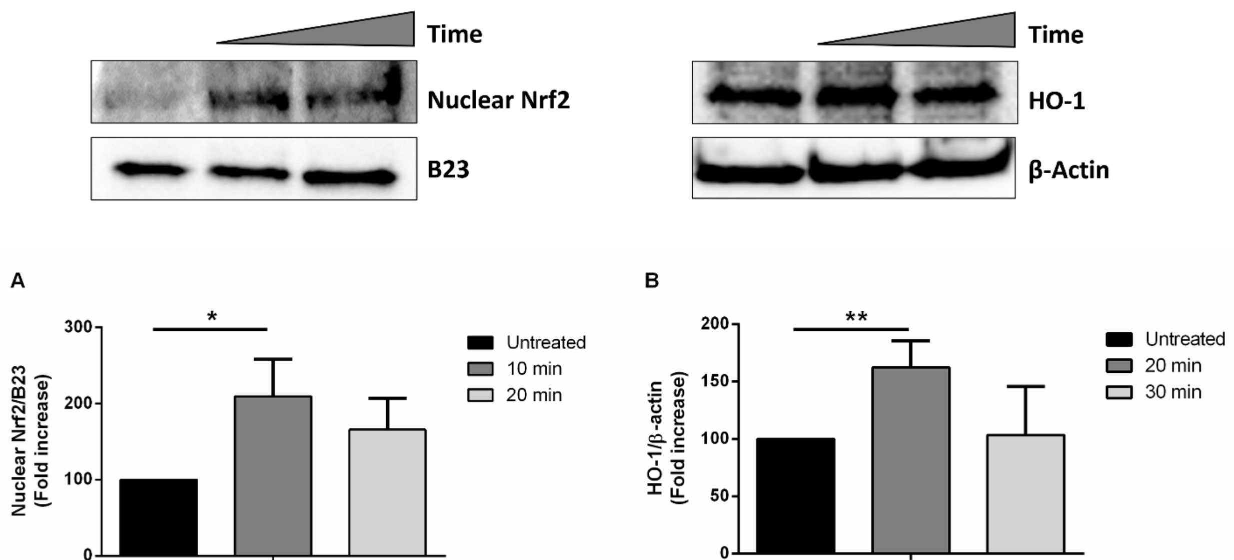

371

372 **Fig. 6 Effect of carotenoid extract on Nrf2 activation.** HaCaT cells were incubated with 90 µg/mL
373 of ethanol extract for different length of times. (**a**) Western blot analysis of nuclear Nrf2 of untreated
374 cells (black bars), after 10 min (dark grey bar) and 20 min (light grey bar) incubation. (**b**) Western
375 blot analysis of cytosolic HO-1 of untreated cells (black bars), after 20 min (dark grey bar) and 30

376 min (light grey bar) incubation. Anti-B23 antibody was used as internal standard for nuclear lysate,
377 whereas anti-β-actin antibody was used for cytosol. Images were quantified by densitometric analysis.
378 Data shown are means ± S.D. of three independent experiments. * Indicates $p < 0.05$ with respect to
379 control cells

380

381 ***Biocompatibility of FAs***

382 Finally, the biocompatibility of palmitic and stearic acids was tested on HaCaT cells. In particular,
383 the FAs obtained at the end of the biorefinery were analyzed following the same procedure used
384 above. As shown in **Fig. 7**, no effect on cell viability was observed at any of the experimental
385 conditions under test, thus indicating the safeness of the isolated FAs.

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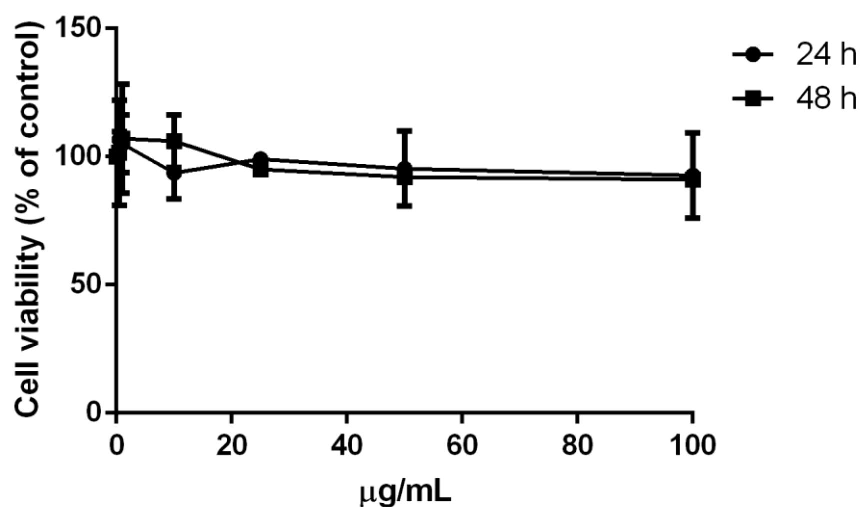

393 **Fig. 7 Biocompatibility of isolated FAs on human keratinocytes.** HaCaT cells were incubated for
394 24 h (black circles) or 48 h (black squares) with increasing concentrations (0.5-100 μg/mL) of FAs
395 isolated from the residual biomass of *P. simplex*. Cell viability is expressed as a percentage of viable
396 cells in the presence of the extracts under test with respect to control cells grown in the absence of
397 the FAs. Data shown are means ± S.D. of three independent experiments

398 **Discussion**

399 In the last years, the use of natural molecules to be used in cosmeceutical field is emerging and the
400 biotech industries are focusing their attention on safe, sustainable and economical natural sources.
401 The blue biotechnology, based on the use of aquatic resources as raw materials, is meeting consumer
402 demands for natural active molecules to be used in cosmetics and cosmeceuticals. In this context,
403 microalgae represent a versatile reservoir of active molecules with many potential applications,
404 ranging from antioxidants (Imbimbo et al. 2019), anticancer (Ferraro et al. 2020) to anti-aging
405 (Khemiri et al. 2023), as its biomass contains an array of valuable metabolites, such as proteins, lipids,
406 polysaccharides pigments and vitamins, all known for their antioxidant, anti-aging and moisturizing
407 activities.

408 However, several drawbacks hinder microalgae large-scale use, which eventually lead to an increase
409 in the overall costs (Imbimbo et al. 2020; Abdur Razzak et al. 2023). A possible solution to lower the
410 costs is the biorefinery approach: a stepwise extraction of more than one product to reduce the
411 environmental footprint (Chanana et al. 2023). This should be combined to eco-friendly extraction
412 techniques to obtain biologically active and safe extracts, in terms of negligible cytotoxicity.

413 Here, an unexplored green microalga strain has been used to isolate different classes of molecules
414 with biological activity. Starting from *Pseudococcomyxa simplex* biomass, carotenoids were extracted
415 as first using a green approach. Lutein, the most abundant one, is a high added carotenoid with
416 reported anticancer, antibacterial, anti-neurodegenerative and anti-inflammatory activities (Desai and
417 Mane 2024), sold at 455,000 €/g. The obtained extract shows a strong antioxidant activity and can
418 activate the Nrf2 pathway, which has been primarily identified as a key player in the antioxidant
419 response. However, from recent studies, the pivotal role of Nrf2 has linked to other key pathways,
420 such as anti-inflammatory and skin-aging (Frantz et al. 2023). Wrinkles, loss of elasticity, hyper-
421 pigmentation, dryness are classical symptoms of skin-aging, mainly due to the activity of key
422 enzymes, such as those involved in the early steps of melanogenesis or in elastin degradation (Shin

423 et al. 2023). Our data strongly support the connection between antioxidants, Nrf2 activation and skin-
424 aging, as the extract shows an inhibitory effect towards tyrosinase and elastase enzymes. Our results
425 are in line with those reported in literature on carotenoids extracted from different strains, such
426 astaxanthin (Mourelle et al. 2017; Dutta et al. 2023) lutein (Jiang et al. 2024) and β-carotene (Yeager
427 and Lim 2019). Accordingly, microalgae extracts are currently being formulated into skin-care
428 products for providing anti-aging, moisturizing, antioxidant, and anti-irritant benefits (Desai and
429 Mane 2024).

430 The lipids isolated from the residual biomass belong to saturated fatty acids, a neglected class of lipids
431 which are now more considered, as they can find application as antibacterial molecules, in cosmetics
432 and in drug delivery (Liberti et al. 2022). Lipid yield obtained from the raw biomass (24%) is in
433 agreement with literature (Santhakumaran et al. 2018), whereas lipid yield from the residual biomass
434 is almost three times lower, probably because of the first extraction with ethanol. However, from the
435 residual biomass, a pure class of fatty acids is obtained by a single purification step. We can
436 hypothesize that lipid yield is affected by the first extraction solvent: the more hydrophobic solvent
437 is used for the first extraction, the lower is the lipid yield obtained from the residual biomass.
438 According to this hypothesis, when an aqueous buffer is used as first extraction solvent, an increase
439 in the lipid yield is observed, as all the lipophilic molecules cannot be extracted by a polar solvent.
440 Moreover, if lipid recovery is preceded by an ethanol extraction, the lipid content decreases, as some
441 class of lipids (those with a lower hydrophobicity) can be co-extracted by ethanol (Imbimbo et al.
442 2019; Liberti et al. 2022) and an increase in SFA content is observed (Liberti et al. 2022). In cosmetics,
443 fatty acids can be used as oily raw materials, as emulsifiers and as softeners because they can deposit
444 between desquamating cells (De Luca et al. 2021). The isolated fatty acids were fully biocompatible,
445 thus suggesting a possible role in cosmetics.

446 The main objective of cosmetic formulations is to use extracts endowed with anti-oxidant, anti-aging
447 and moisturizing potential and microalgae fulfill these requirements and they also represent a

448 sustainable vegan alternative to replace potentially harmful chemicals, traditionally used in skin care
449 products. Algae molecules can protect from aging, UV and oxidative stress, but only few strains are
450 commercialized. Currently, molecules or extracts from *Chlorella vulgaris*, *Haematococcus pluvialis*,
451 *Dunaliella salina*, *Nannochloropsis oculata* and *Phaeodactylum tricornutum* are normally employed,
452 so that the exploration of untapped species is now mandatory. This study represents a milestone but
453 also a starting point for process improvement. Indeed, biomass concentration, advances in extraction
454 technologies, formulation strategies and a detailed cost analysis are expected to accelerate the
455 adoption of *P. simplex* in the cosmetics industry.

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459 **Ethics declarations**

460 **Ethics approval**

461 This article does not contain any studies with human participants or animals performed by any of the
462 authors.

463 **Conflict of interest**

464 The authors declare no competing interests.

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467 **E.G.** investigation, data analysis and writing original draft**; A.F.** investigation and writing original
468 draft; **G.A.R.** Investigation, writing original draft**: H.A. Investigation; E.I.** Methodology, review-
469 editing; **M.C.D.M.** Investigation, writing original draft; **A.Z.** Methodology, review-editing;
470 **D.M.M** Conceptualization, Supervision, and Writing original draft, review-editing. All authors read
471 and approved the manuscript.

472 **Data availability**

473 All data generated during this study are included in this published article and its supplementary
474 information file.

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