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Bio-Guided Fractionation and Molecular Networking of Three Marine Microalgae for Anti-Inflammatory and Antioxidant Applications in Cosmetics

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1. Introduction

Microalgae exhibit remarkable phylogenetic diversity and play a major role on Earth as primary producers in aquatic food webs and major contributors to the global carbon cycle through photosynthesis [1, 2]. Although approximately 18,000 marine species of microalgae have been described to date [3], this number is likely a considerable underestimate, with some estimates suggesting several orders of magnitude more [4, 5]. This diversity is paralleled by a vast natural chemical repertoire, including numerous families of bioactive secondary metabolites with pharmaceutical and cosmetic relevance [6, 7, 8].

This diversity of bioactive compounds makes microalgae a highly valuable natural resource and has attracted growing interest from various industrial sectors, particularly cosmetics [9]. In this context, we selected three marine microalgal species for their promising potential in cosmetics: *Dunaliella salina*, *Tetraselmis suecica*, and *Cylindrotheca fusiformis*.

Dunaliella salina is best known for its ability to hyperaccumulate β-carotene under stress conditions [10, 11]. This bioactive carotenoid exhibits antioxidant and anti-inflammatory activities and is particularly valued in skincare for preventing skin aging and protecting against sun damage [12,13]. *Tetraselmis suecica*, is also a rich source of bioactive compounds relevant to skin health, including α-tocopherol, eicosapentaenoic acid (EPA), lutein, and β-carotene [14, 15, 16]. *Cylindrotheca fusiformis* is recognized for its dual production of two valuable bioactive compounds: fucoxanthin and EPA [17, 18, 19]. In addition to their known bioactive compounds, several studies have reported antioxidant and anti-inflammatory activities associated with extracts of these microalgae: *Dunaliella salina* [20-22], *Tetraselmis suecica* [14, 23, 24], and *Cylindrotheca fusiformis* [25].

In this study, we aimed at evaluating the antioxidant and anti-inflammatory potential of ethanolic crude extracts from *D. salina*, *T. suecica*, and *C. fusiformis* using *in vitro* skin cell models, and identifying the metabolites responsible for these activities. For this purpose, a bio-guided fractionation process was employed. Since *C. fusiformis* was the least studied of the three

models and its fractions exhibited promising bioactivities, an additional bio-guided fractionation step was carried out on the most relevant fractions.

This approach uncovered an unexpected family of compounds in *C. fusiformis* that exhibit potential anti-inflammatory properties, making them promising candidates for cosmetic applications.

2. Materials and Methods

Microalgal biomass

Cylindrotheca fusiformis (CCAP 1017/14) and *Dunaliella salina* (CCAP 19/18) were obtained from the Culture Collection of Algae & Protozoa, and *Tetraselmis suecica* (AC254) from Algo-bank. *Cylindrotheca*, *Dunaliella*, and *Tetraselmis* were cultured in F2-Si, F2, and Conway media, respectively, using filtered and sterilized natural seawater (salinity 35.5‰) from the Bay of Quiberon (France). Cultures were maintained at 18–25°C under phototrophic conditions with white LED light. Biomass was harvested by centrifugation, freeze-dried, ground to a fine powder, and stored at –20°C.

Extraction and fractionation

Crude extracts: One gram of each microalgal biomass was extracted three times with 33 mL of ethanol (EtOH), using vortexing and ultrasonic treatment (15 min, 25 kHz). The extracts were combined and concentrated to obtain crude extracts at a concentration of 100 mg mL⁻¹.

First fractionation (fractions): Semi-preparative reversed-phase chromatography of the three crude extracts was performed using an Uptisphere® C18-TF column (250 mm × 10 mm, 5 µm, Interchim) on an HPLC-UV-DAD system (1200 Series, Agilent Technologies) equipped with a fraction collector. A binary mobile phase—solvent A (Milli-Q water) and solvent B (acetonitrile, ≥99.9%)—was used at a rate of 4 mL min⁻¹ with an injection volume of 500 µL. The elution gradient was: 20% B for 2 min, linear ramp to 95% B over 30 min, held for 15 min, decreased to 30% B in 2 min, and equilibrated for 8 min. UV detection was performed at 210 and 256 nm and within a 200–300 nm scan range (DAD). Fractions were collected every 2 min and pooled based on the 210 nm chromatogram, yielding six fractions per microalga (F1–F6). A total of 250 mg crude extract per microalga was fractionated over five injections. Pooled fractions were concentrated in EtOH to 10 mg mL⁻¹ and stored at –20°C.

Second fractionation (sub-fractions) on *C. fusiformis*: The extraction and first fractionation steps were repeated on 8 g of *Cylindrotheca* dry biomass to isolate fractions F2, F3, and F4 in sufficient amounts for further purification. The same apparatus and mobile phases were used, with customized elution gradients for finer separation: F2: 30% B for 10 min, 30–60% in 30 min, 60% for 5 min, 60–95% in 5 min, 95% for 20 min, 95–30% in 5 min, 30% for 10 min; F3: 55% B for 10 min, 55–75% in 20 min, 75% for 5 min, 75–95% in 5 min, 95% for 20 min, 95–55% in 5 min, 55% for 10 min; F4: 60% B for 10 min, 60–95% in 40 min, 95% for 15 min, 95–60% in 5 min, 60% for 10 min. Two injections were performed per fraction, with 26 mg, 80 mg, and 117 mg of crude extract injected for F2, F3, and F4, respectively. Sub-fractions were collected every 2 min and pooled based on the 210 nm chromatogram, yielding 4 sub-fractions for F2, 5 for F3, and 6 for F4. Each pooled sub-fraction was concentrated in EtOH to 2 mg mL⁻¹ and stored at –20°C in the dark.

Targeted analyses

Pigment content was analyzed by high-performance liquid chromatography coupled with a UV diode-array detector (HPLC-UV-DAD). Fatty acid content was analyzed after sample evaporation and transesterification into fatty acid methyl esters (FAMEs), following the 'FAME Preparation' protocol. Hexane extracts were injected into a gas chromatography (GC) instrument (TRACE™ 1310, Thermo Scientific™), equipped with a TR-Wax GC fused silica capillary column (30 m × 0.25 mm × 0.25 µm, 260W142P), and coupled to a flame ionization detector (FID).

Untargeted analysis, dereplication and molecular networking

Liquid Chromatography-High Resolution Tandem Mass Spectrometry (LC-HRMS/MS) analyses were conducted. All samples were diluted in ethanol to a concentration of 1 mg extract mL⁻¹. Injections were randomized, and blanks were run at the beginning of the sequence for data curation. MS² data were converted from Agilent .d to .mzXML format using MS-Convert (ProteoWizard 3.0), then processed with MZmine 2 (v2.53). Resulting .mgf file was used to generate a feature-based molecular network (FBMN) on GNPS (<http://gnps.ucsd.edu>), with the following parameters: MS1 tolerance 0.5 Da, MS2 tolerance 0.2 Da, cosine score ≥0.7, ≥6 shared fragments, minimum cluster size 1, no MS-cluster algorithm, and TopK = 1,000. MS/MS spectra were matched against GNPS libraries for dereplication. The FBMN was visualized in Cytoscape (v3.10.3) using .selfloop and .csv files. Blanks were excluded, nodes were color-coded by average peak area across extract/fractions/sub-fractions, and edge width scaled with cosine score (thin at 0.7, thick at 1). The GNPS molecular networking job is accessible at: <https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=5fbba35d43ef451badd488410c63417a>.

Skin cell models:

Antioxidant assay (intracellular ROS levels): Normal Human Epidermal Keratinocytes (NHEK), pre-incubated in presence of 20µM DCFH-DA probe for 1h, were treated by antioxidant control (Vitamin C, 500 µM; Quercetin, 20 µM) or test samples in presence of H₂O₂ (400 µM) to induce oxidative stress. Plates were incubated for 45 min before fluorescence measurement (485 nm excitation/535 nm emission).

Anti-inflammatory assay (IL-8 and TNF-α release): NHEK were treated with test samples or anti-inflammatory control (Dexamethasone, 0.1 µM). After 24 h incubation, inflammatory stress was induced by directly adding PMA (Phorbol-Myristate-Acetate) to reach a final concentration of 0.1 µg mL⁻¹ per well. Plates were incubated for an additional 16 h. Interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-α) release were quantified in supernatants by ELISA assays.

3. Results

3.1. Biological activity of the crude extracts

Dose-dependent antioxidant effects were observed for the three microalgal extracts (Figure 1a). Notably, some effects rivaled those of the antioxidant controls (Quercetin and Vitamin C), with *Tetraselmis* at C1 significantly inhibiting H₂O₂-induced ROS levels and even reducing basal ROS levels in the cells (-115%).

No anti-inflammatory effect was observed toward IL-8 (not shown). In contrast, significant inhibition of TNF- α release was observed at the highest concentration (C'1), with inhibition of -95% and -70% for *Tetraselmis* and *Cylindrotheca*, respectively (Figure 1b).

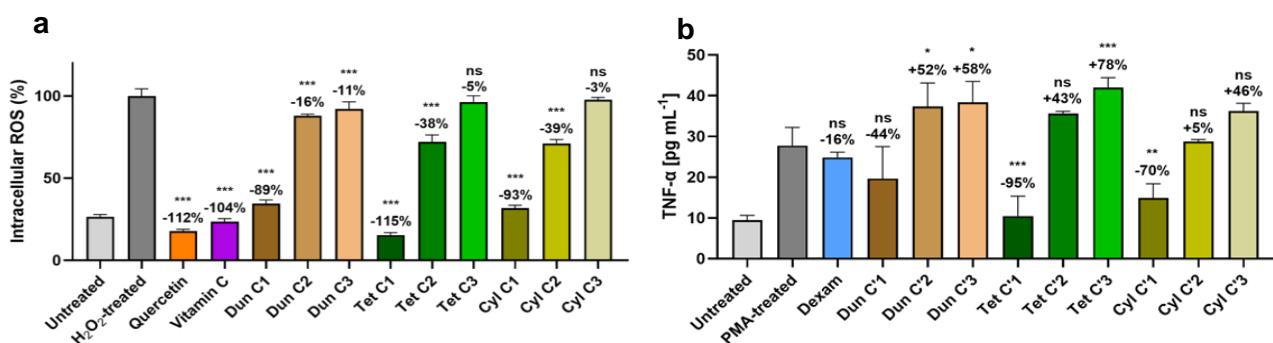


Figure 1. Antioxidant and anti-inflammatory activities of ethanolic crude extracts from *Dunaliella* (Dun), *Tetraselmis* (Tet), and *Cylindrotheca* (Cyl) on NHEKs. (a) Intracellular ROS levels measured using the DCFH-DA in H₂O₂-stressed NHEKs. (b) TNF- α release measured by ELISA in NHEK supernatants. Statistical significance compared with H₂O₂ and PMA controls in both assays is indicated: *p < 0.05, **p < 0.01, ***p < 0.001, ns: not significant (one-way ANOVA followed by Dunnett's test).

3.2. Studies on the three micro-algae fractions

The same NHEK-based bioassays as in 3.1 were performed on each of the six fractions obtained from the first fractionation of crude extracts from *Dunaliella*, *Tetraselmis*, and *Cylindrotheca*. ROS-scavenging effects were observed for all fractions, ranging from -85% (TF3) to -24% (DF5), while anti-inflammatory effects were limited to 2–4 fractions per alga, ranging from -28% (CF1) to -7% (TF4) for IL-8 release and -45% (CF2) to -10% (DF3) for TNF- α release (Fig. 2). No clear trends were observed between the bioactivity of fractions and their pigment or omega-3 fatty acid content, except that some pigment- and omega-3-rich fractions showed pronounced pro-inflammatory effects in each microalga (e.g., DF6, TF5, TF6, and CF4).

Samples	Bioactivity on NHEKs			Metabolite Profiles of Samples (μ g/mg Dry Extract)										
	Antioxidant	Anti-inflammatory		Pigments					n-3 PUFA					
		ROS (%)	IL-8 (%)	TNF- α (%)	Phide a	Fuco-OH	Fuco	Lut	Chl b	Chl a	Phytin a	β -Car	ALA	EPA
Dunaliella	D CE	100%	-89	15	-44	7.9		2.7	2.5	5.7	50	0.85	241	
	DF1	69%	-54	-19	26	2.2								
	DF2	2%	-54	-20	-11									
	DF3	4%	-46	-20	-10	2.4								
	DF4	3%	-52	-8	31	2.4								
	DF5	12%	-24	14	42	0.20	1.1				0.13			
Tetraselmis	TF1	29%	-45	-23	9									
	TF2	3%	-49	-14	-43	0.65					0.11	0.18		
	TF3	6%	-85	-8	-24	17								
	TF4	3%	-62	-7	-25						5.8	0.22		
	TF5	15%	-59	44	60						23	6.4	83	20
	TF6	44%	-65	40	81						10	25	86.0	25
Cylindrotheca	CE	100%	-115	16	95						4.5	16	54	12
	CF1	29%	-45	-23	9						2.3	2.3		
	CF2	3%	-49	-14	-43	0.65								
	CF3	6%	-85	-8	-24	17								
	CF4	3%	-62	-7	-25									
	CF5	15%	-59	44	60									

Figure 2. Summary of antioxidant and anti-inflammatory activities on NHEKs, with pigment and omega-3 fatty acid profiles of crude extracts and fractions from *Dunaliella*, *Tetraselmis*, and *Cylindrotheca*. Abbreviations: Crude extract (CE), Pheophorbide a (Phide a), Fucoxanthinol (Fuco-OH), Fucoxanthin (Fuco), Chlorophyll a/b (Chl a/b), Pheophytin a (Phytin a), β -Carotene (β -Car), omega-3 polyunsaturated fatty acids (n-3 PUFA), α -Linolenic acid (ALA), Eicosapentaenoic acid (EPA), and Docosahexaenoic acid (DHA).

Cylindrotheca fusiformis fractions CF2, CF3, and CF4 were selected for a second fractionation: CF2 for its anti-inflammatory effects on cytokine release (-45% TNF- α and -14% IL-8), CF3 for its antioxidant effect (-81% ROS), and CF4 for its relatively high content of Fuco-OH, Fuco, EPA, and DHA.

3.3. Studies on *C. fusiformis* sub-fractions

As with the fractions, ROS-scavenging effects were observed for all sub-fractions from *C. fusiformis*, ranging from -33% (F4F'2) to -13% (F4F'1) (Figure 3). Anti-inflammatory effects were predominantly present in sub-fractions from CF2 (i.e., F2F'1 to F2F'4), ranging from -29% (F2F'2) to -12% (F2F'4) for IL-8 release and -80% (F2F'2) to -9% (F2F'2) for TNF- α release.

Sub-fraction F3F'1, accounting for more than half of CF3's mass, exhibited potent anti-IL-8 and anti-TNF- α effects, with reductions of -67% and -78%, respectively. Overall, sub-fractions with minimal or absent pigment and omega-3 fatty acid content showed the strongest anti-inflammatory activity (i.e., F2F'2 to F2F'4 and F3F'1). These sub-fractions of interest were selected and integrated with *C. fusiformis* fractions for the molecular networking-based dereplication approach (Figure 4).

Frac	Sub-frac	Mass %	Bioactivity on NHEKs			Metabolite Profiles ($\mu\text{g}/\text{mg}$ Dry Extract)				
			Anti-inflammatory		Antioxidant	Pigments			n-3 PUFAs	
			IL-8 (%)	TNF- α (%)	ROS (%)	Phide α	Fuco-OH	Fuco	EPA	DHA
CF2	F2F'1	68%	-25	-9	23					0.085
	F2F'2	10%	-29	-80	20					1.63
	F2F'3	18%	-18	-43	28					
	F2F'4	4%	-12	-68	18					
CF3	F3F'1	51%	-67	-78	16				1.2	0.16
	F3F'2	36%	-21	20	26	0.99			82	0.11
	F3F'3	1%	5	-2	29	94	0.37		28	13
	F3F'4	7%	4	-9	25	37	2.0		1.5	
	F3F'5	5%	21	21	18	69		0.11	28	1.0
CF4	F4F'1	28%	2	10	13	1.5	0.45		21	6.2
	F4F'2	6%	11	49	33	0.86	146		6.2	15
	F4F'3	13%	18	13	20	0.80	12		1.1	3.3
	F4F'4	23%	7	23	19	1.3		33	0.10	
	F4F'5	19%	30	32	31	22		9.1	0.44	2.5
	F4F'6	10%	45	16	25	2.8			3.7	0.38

Figure 3. Summary of antioxidant and anti-inflammatory activities on NHEKs, with pigment and omega-3 fatty acid profiles of sub-fractions from *Cylindrotheca fusiformis*.

The FBMN consists of 2,184 nodes, containing 5 major clusters (>20 nodes) and 25 small clusters (5–20 nodes), representing 35% of the total nodes. A total of 118 features were annotated across the full network using the GNPS libraries. Only major clusters are shown (Fig. 4), highlighting molecular families such as carotenoids, pheophorbides, N-fructosyl amino acids, and N-acyl amino acids. Additional putative compounds, notably steroids, lipids, phenolic compounds, and fatty acids, were scattered across the network. The relative abundance of features within the crude extract (CE), fractions (Fs), and sub-fractions (F's) is indicated by color-coded pie charts.

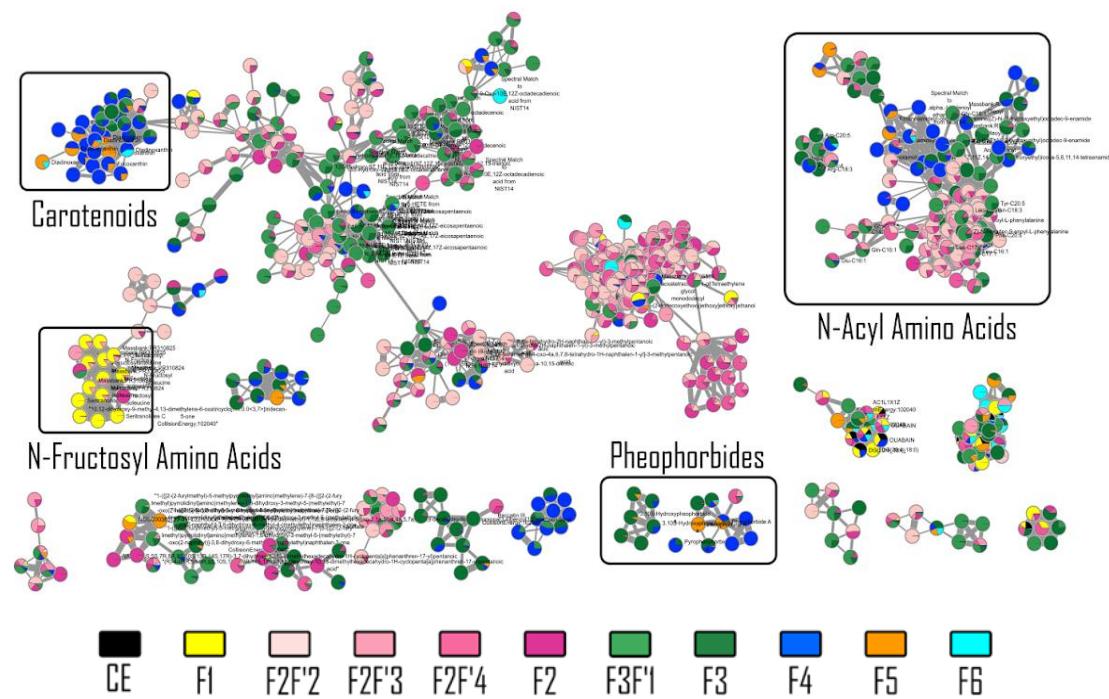


Figure 4. Major clusters of the feature-based molecular network (FBMN) of *C. fusiformis*, including fractions (F1–F6) and selected sub-fractions (F2F'2 to F2F'4, and F3F'1). Each node represents a precursor ion, with a color-coded pie chart indicating its relative abundance in the crude extract, fractions, and sub-fractions. Edge width represents MS/MS similarity (cosine score ≥ 0.7) between two features. Features matching the GNPS library are labeled on nodes and were used for putative annotation of some molecular families framed and highlighted in the network.

Among the clusters of bioactive sub-fractions (F2F'2 to F2F'4, and F3F'1), two were comparatively well-annotated, revealing a variety of N-acyl amino acids (Figure 5, Table 1). Twenty-two occurrences of N-acyl amino acid were found among the 118 dereplicated features, with 21 present in both clusters, including 11 with a Δppm below 5 .

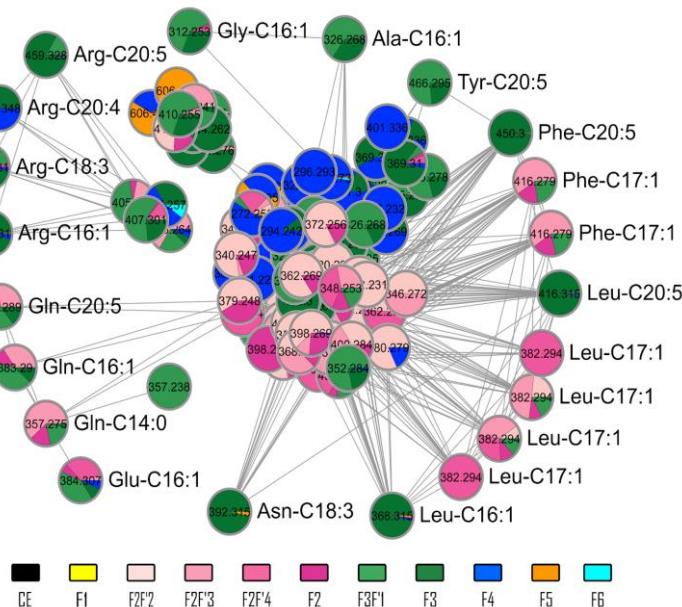


Figure 5. Annotated clusters of N-acyl amino acids putatively identified in *C. fusiformis* using the GNPS library. Nodes are labeled with precursor ion m/z values and contain color-coded pie charts showing their relative abundance across the extract, fractions, and sub-fractions. For clarity, only putative NAAA matches are annotated.

Table 1. List of the putative N-acyl amino acids in Fig. 7 with $\Delta ppm < 5$, all these hits were [M+H]⁺ adducts.

	Compound name	Molecular formula	Measured m/z	RT (min)	Δppm
Leu-C20:5	N-eicosapentaenoyl-L-leucine	C26H41NO3	416.3154	10.05	1.5
Gly-C16:1	N-palmitoleoyl glycine	C18H33NO3	312.2527	9.16	1.0
Leu-C16:1	N-palmitoleoyl-L-leucine	C22H41NO3	368.3153	10.34	1.9
Gln-C16:1	N-palmitoleoyl-L-glutamine	C21H38N2O4	383.2897	8.34	0.8
Ala-C16:1	N-palmitoleoyl-L-alanine	C19H35NO3	326.2678	9.45	3.7
Tyr-C20:5	N-eicosapentaenoyl-L-tyrosine	C29H39NO4	466.2949	9.07	0.3
Phe-C20:5	N-eicosapentaenoyl-L-phenylalanine	C29H39NO3	450.3002	10.02	0.5
Gln-C20:5	N-eicosapentaenoyl-L-glutamine	C25H38N2O4	431.2892	8.26	1.8
Arg-C16:1	N-palmitoleoyl-L-arginine	C22H42N4O3	411.3312	7.73	4.4
Arg-C20:4	N-arachidonoyl-L-arginine	C26H44N4O3	461.3479	8.11	2.4
Gln-C14:0	N-myristoyl-L-glutamine	C19H36N2O4	357.2747	8.04	0.9

4. Discussion

Crude ethanolic extracts

In a first step, the study explored the antioxidant and anti-inflammatory properties of crude ethanolic extracts from three microalgal species: *Dunaliella salina*, *Tetraselmis suecica*, and *Cylindrotheca fusiformis*.

All extracts exhibited significant and dose-dependent antioxidant effects. These effects were expected, as similar results have been previously reported for various extracts from these microalgal species [26-32]. Sansone *et al.* [14] reported that a hydroethanolic *T. suecica* extract exhibited antioxidant potential comparable to that of vitamin C (ascorbic acid) in reducing free radicals. In line with these results, *T. suecica* showed similar efficacy to vitamin C in reducing intracellular ROS levels in our study.

However, the anti-inflammatory effects were less pronounced, with notable reductions in TNF- α release only at high extract concentrations, and no significant impact on IL-8 release. In contrast, several studies have reported significant reductions in pro-inflammatory cytokine levels following treatment with extracts derived from the same microalgal models. These discrepancies in cytokine modulation could be attributed to differences in cell models, extract composition, or concentration-dependent effects.

Finally, the pigment and omega-3 fatty acid profiles of the crude microalgal extracts were consistent with previously reported data, and confirmed the presence of known bioactive compounds such as EPA, fucoxanthin, lutein, or β -carotene [14, 18, 33, 34]. Altogether, these findings support the use of a bio-guided fractionation approach to identify the metabolites responsible for the observed bioactivities.

Fractions and subfractions

The fractions and sub-fractions obtained from the fractionation process were analyzed for their antioxidant and anti-inflammatory properties, as well as their pigment and omega-3 fatty acid content. Antioxidant activity was present in all fractions, with ROS-scavenging effects ranging

from -85% to -24%, making it challenging to pinpoint specific fractions for further study based solely on this activity. This trend was also seen in sub-fractions, which generally showed weaker antioxidant effects. The varied antioxidant metabolites in microalgae, both hydrophilic and lipophilic, likely contributed to the broad distribution of antioxidant activity. Interestingly, the most potent antioxidant fractions, TF3 and CF3, lacked pigments and fatty acids, suggesting other compounds like phenolic substances might be responsible. Some studies support a link between antioxidant properties and phenolic content, while others do not, indicating other compounds may also play a role.

In contrast, variations in cytokine modulation (IL-8 and TNF- α) were more distinct, with both anti- and pro-inflammatory responses observed. Anti-inflammatory effects were limited to a few hydrophilic fractions per microalga, lacking carotenoids and omega-3 fatty acids. This was confirmed in *C. fusiformis* sub-fractions, where anti-inflammatory activity was seen in the more hydrophilic ones, despite expectations that compounds like fucoxanthin and EPA would show such effects. Testing pure standards of these compounds could clarify their contributions to the observed bioactivities. Following the fractionation steps, the resulting fractions and sub-fractions were tested for antioxidant and anti-inflammatory activities and characterized for their pigment and omega-3 fatty acid content.

Molecular network and N-acyl amino acids

The study identified promising anti-inflammatory effects in certain sub-fractions from the *C. fusiformis* extract, with significant reductions in TNF- α and IL-8 release. These effects were not linked to known pigments or omega-3 fatty acids. Instead, a molecular network analysis revealed the presence of N-acyl amino acids (NAAAs), a novel finding in this context. NAAAs are bioactive lipids known for their diverse physiological roles, including anti-inflammatory and immunomodulatory activities [38-41]. They are typically found in mammals but less documented in plants, making their detection in *C. fusiformis* noteworthy. The structural diversity of NAAAs, influenced by variations in fatty acid chains and amino acid head groups, affects their bioactivity. Some NAAAs identified in *C. fusiformis* share structural features with those known for strong anti-inflammatory effects. These findings suggest that NAAAs in *C. fusiformis* could be valuable for cosmetic applications due to their multifunctional properties. However, the annotations are tentative, and further purification and structural elucidation are needed to confirm the active components.

5. Conclusion

The study highlights the antioxidant and anti-inflammatory potential of three marine microalgae: *Dunaliella salina*, *Tetraselmis suecica*, and *Cylindrotheca fusiformis*, particularly on human epidermal keratinocytes. While strong antioxidant effects were noted, the anti-inflammatory effects varied. Through bio-guided fractionation, promising fractions were identified, but no clear link was found between pigments or omega-3 fatty acids and these bioactivities. Further analysis of *C. fusiformis* revealed N-acyl amino acids, a novel finding in diatoms, potentially contributing to the observed anti-inflammatory effects. These compounds, known for modulating inflammation and having beneficial properties, could be valuable for cosmetic applications. Future research could explore similar compounds in *D. salina* and *T. suecica*, enhancing the understanding of these findings and their potential in sustainable cosmetics.

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