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The green microalga *Tetraselmis suecica* reduces oxidative stress and induces repairing mechanisms in human cells

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Green microalgae contain many active pigments such as carotenoids having antioxidant and protective activity on human cells. Here we investigate the biological activity of an ethanol/water extract of the marine green microalga *Tetraselmis suecica* containing high levels of carotenoids such as the xanthophylls lutein, violaxanthin, neoxanthin, antheraxanthin and loroxanthin esters. This extract has a strong antioxidant and repairing activity in the human lung cancer cell line (A549) as shown by the increased expression of dehydrocholesterol reductase-24 (DHCR24) and prostaglandin reductase 1 (PTGR1) genes and proteins. The extract also reduces prostaglandin E₂ (PGE₂) levels in cells damaged by H₂O₂ and has tissue repairing effects on reconstructed human epidermal tissue cells (EpiDerm™) indicating a potential cosmeceutical activity of this microalgal species.

Reactive oxygen species (ROS) have been linked to the pathogenesis of several human diseases such as atherosclerosis, diabetes mellitus, chronic inflammation, neurodegenerative disorders and many types of cancers. ROS species can be partially neutralized by antioxidant compounds that can reduce the risk of many diseases related to oxidative stress¹. Hence, consumer preference for natural products is increasing the interest in finding new antioxidants from natural sources because synthetic products can cause potential long term toxic effects². Most, if not all, commercially available natural antioxidants are derived from terrestrial plants (e.g. rosemary, tea, coffee, grape seeds, tomato and cocoa). Many of these antioxidants are carotenoids that are a class of more than 700 naturally occurring pigments synthesized by plants, algae, and photosynthetic bacteria. Carotenoids are known to be potent physical and chemical quenchers of singlet oxygen (¹O₂) and scavengers of other reactive oxygen species (ROS). However, the exact mechanisms underlying the protective function and specific molecular targets of carotenoids *in vivo* and *in vitro* are still poorly understood³.

Tetraselmis suecica is a marine green microalga belonging to the class Chlorophyceae, widely used in aquaculture for the feeding of mollusks and crustacean larvae⁴ and as a probiotic in fish⁵. *T. suecica* is rich in vitamin E, carotenoids, chlorophyll, and tocopherols⁶ and has been suggested as a food supplement in human and animal diets⁷. The total pigment extract from *T. suecica* has been patented for its ability to enhance dermal pigmentation, reduce psoriasis lesions and increase hair growth⁷. Here we investigate the potential biotechnological application of this species studying the protective role at molecular level on human anaplastic cells and tissues. To this aim, we characterize the pigment content of an ethanol/water extract of *T. suecica* and investigate the antioxidant and protective effects of this extract against oxidative damage. We show that this extract has a strong antioxidant and cell repairing activity in a human lung cancer cell line (A549), an *in-vitro* model that is often used to study antioxidant effects⁸. In particular, this total extract targets the expression of dehydrocholesterol reductase-24 (DHCR24) and prostaglandin reductase 2 (PTGR2) genes and proteins, and reduces the levels of prostaglandin E₂ (PGE₂).

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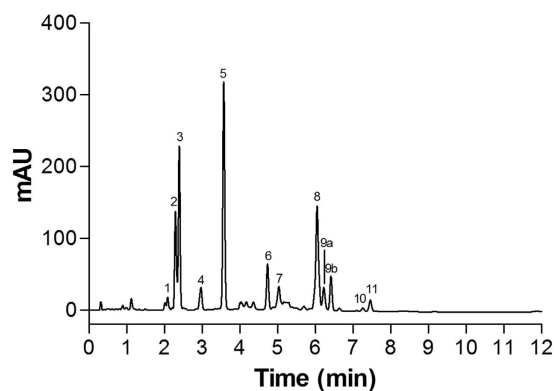


Figure 1. HPLC chromatogram of pigments from an ethanol/water extract of the green microalga *Tetraselmis suecica*.

Peak	Retention time (min)	Pigment	Abbreviation	Absorbance maxima (nm)
1	2.08	Violaxanthin-like	Viola-like	415–440–469
2	2.29	Neoxanthin	Neo	413–436–465
3	2.39	Violaxanthin	Viola	415–440–469
4	2.97	9'-cis-Neoxanthin + Antheraxanthin	c-Neo Anth	413–436–465 422–447–472
5	3.58	Lutein	Lut	418–444–474
6	4.74	Loroxanthin dodecenoate	Loro- <i>d</i>	420–448–473
7	5.04	Chlorophyll <i>b</i>	Chl <i>b</i>	457–650
8	6.05	Chlorophyll <i>a</i>	Chl <i>a</i>	430–618–665
9a	6.23	Chlorophyll <i>a'</i>	Chl <i>a'</i>	430–618–665
9b	6.42			
10	7.26	γ -Carotene	γ -Car	436–462–490
11	7.46	α -Carotene	α -Car	425–449–476

Table 1. Peak identification, retention time, abbreviations and online spectral characteristics.

Finally, the cell repairing effect of this extract is demonstrated in *in vitro* reconstructed human epidermal tissue cells (EpiDerm™) indicating a potential cosmeceutical activity of this microalgal species.

Results

The high performance liquid chromatography (HPLC) pigment profile of the ethanol/water extract of *T. suecica* revealed porphyrin pigments, chlorophyll *a* and *b*, α - and γ -carotene and xanthophyll pigments such as lutein, loroxanthin dodecenoate, violaxanthin, neoxanthin, 9'-cis-neoxanthin and antheraxanthin (Fig. 1, Table 1). The pigments were identified by diode array (DAD) spectroscopy and comparing their visible absorption spectra with authentic standard.

Results were also supported by HPLC-PDA-MS/MS data (Supporting Information, Figure S1, Table S1). Other xanthophyll pigments, such as loroxanthin and zeaxanthin, which are usually found in green algae, were not observed. Loroxanthin decenoate was tentatively identified only by LC-MS/MS analysis. Xanthophylls constituted almost 79% of the total pigments identified, and, within the group, lutein was the most abundant, reaching concentrations comparable to that of chlorophyll *b* (Chl *b* 31% of Chl *a*, Lutein 33% of Chl *a*, Fig. 2). Neoxanthin and violaxanthin pigments showed a percentage over Chl *a* of about 16%, whereas loroxanthin dodecenoate a percentage of 8%. This ethanol/water extract (herewith referred to as extract) exhibited marked reducing activity toward radical species when the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability was tested. Addition of extract concentrations of 25, 50 and 100 μ g resulted in a dose-dependent reduction (21.5%, 52.0% and 97.7%, respectively) of the purple radical DPPH into the yellow reduced form. This activity was significantly stronger than the positive control, α -Tocopherol, tested at the same concentrations (Table 2).

Lung adenocarcinoma (A549) cells treated with different concentrations of the extract for 24 and 48 h were not affected at any of the concentrations tested (2, 5, 10, 25, 50, 100, 200 μ g ml⁻¹) except for A549 cells at the highest concentration, which induced a slight reduction in cell viability (80 and 81% cell viability, at 400 μ g ml⁻¹, at the two incubation times, Fig. 3A).

In order to assess the antioxidant effects of the extract we induced an oxidative stress on A549 cells with hydrogen peroxide (H₂O₂). First, we treated A549 cells with a wide range of H₂O₂ concentrations (0.3, 3, 30 and 300 mM) to determine the half maximal Inhibitory Concentration (IC₅₀) dose for pretreatment of cells, before recovery experiments with extract; the IC₅₀ dose was established as 30 mM after 24 h and 48 h of treatment

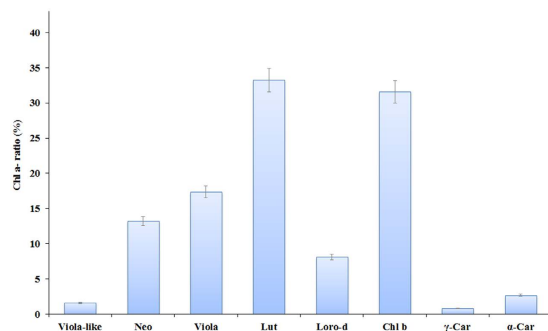


Figure 2. Pigment content of *Tetraselmis suecica* ethanol/water extract. Content data of 9'-*cis*-Neoxanthin and Antheraxanthin are missing due to their co-elution. Data are reported as percentage of pigment/chlorophyll a ratio.

Extract concentration	($\mu\text{g/ml}$)	Optical density (OD517 nm)	Inhibition percentage (IP) of DPPH radical
ODT0-ODTS			
Tetraselmis suecica	0	0.000 \pm 0.001	0.0 \pm 0.789
	50	0.0655 \pm 0.029	21.1 \pm 2.87*
	100	0.081125 \pm 0.034	52.00 \pm 3.44*
	200	0.111 \pm 0.051	97.72 \pm 5.09*
	0	0.000 \pm 0.001	0.0 \pm 0.789
α -Tocopherol	50	0.0125 \pm 0.052	5.98 \pm 5.21
	100	0.026 \pm 0.035	12.36 \pm 1.10*
	200	0.040 \pm 0.013	26.00 \pm 1.3*

Table 2. Radical scavenging capacity (RSC, %) of *Tetraselmis suecica* ethanol/water extract on DPPH free radical. Values are reported as percentage versus a blank and are the mean \pm SD of three independent samples analyzed three times. Asterisks denote significant increases in measured radical scavenging activity * $p \leq 0.05$ versus control.

(Fig. 3B). We then treated A549 cells with 30 mM of H_2O_2 for 1 h, inducing H_2O_2 oxidative stress, and added the extract to cells. The extract induced a significant recovery effect on H_2O_2 stressed A549 cells after 48 h of treatment. Histograms in Fig. 3C show the effect of treatment with H_2O_2 (injury treatment) and extract. Treatment with H_2O_2 resulted in 36% cell viability after 48 h, whereas addition of extract induced a proliferation of A549 cells, with a cell viability increase of 143, 114, 125, 112, 133, 126, 114 and 148% for all concentrations tested (2, 5, 10, 25, 50, 100, 200 and 400 $\mu\text{g ml}^{-1}$) with respect to the negative control. Within the experimental error, there was a plateau effect already at 2 $\mu\text{g ml}^{-1}$ of the extract and a further increase of this concentration did not affect cell viability.

The expression of genes involved in oxidative stress and repairing pathways (Supplementary information, Table S2) were analyzed in A549 cells treated with 100, 200 and 400 $\mu\text{g ml}^{-1}$ of extract alone (Fig. 4D) and 100, 200 and 400 $\mu\text{g ml}^{-1}$ of extract after 1 h of exposure to 30 mM of H_2O_2 (Fig. 4A,B and C respectively) since lower extract concentrations did not induce changes in gene and protein expression. Gene expression results are reported after 2 h of treatment since several factors implicated in oxidative damage repairing pathways were already expressed and activated after this time interval. Control genes for real-time qPCR were actin-beta (ACTB), beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase (HPRT1) and ribosomal protein large P₀ (RPLP₀), the expression of which remained constant. For gene expression studies, we chose the three highest concentrations (100, 200 and 400 $\mu\text{g ml}^{-1}$) that showed strong repairing activities even if we observed a slight cytotoxicity (about 20%) at the two highest concentrations (200 and 400 $\mu\text{g ml}^{-1}$). Notwithstanding the toxicity, there was a significant dose-dependent activation of specific oxidative stress response mechanisms without activating genes involved in cell death programs with all three concentrations. This suggests that the slight cytotoxicity did not inhibit the repairing activity of the extract.

24-dehydrocholesterol reductase (DHCR24) was down-regulated only at 200 $\mu\text{g ml}^{-1}$ (2.2-fold change). Whereas at 400 $\mu\text{g ml}^{-1}$ all the following genes were down-regulated: glutathione peroxidase 4 (GPX4, 2.0-fold change), glutathione S-transferase pi 1 (GSTP1, 2.0-fold change), peroxiredoxin 5 (PRDX5, 2.6-fold change), sirtuin 2 (SIRT2, 5.18-fold change) and heat shock protein alpha class A member 1 (HSP90AA1, 90 kDa, 4.3-fold change). At the same concentration, there was an up-regulation of forkhead box M1 (FOXMI, 2.71-fold change), superoxide dismutase (SOD2, 2.0-fold change) and prostaglandin reductase 1 (PTGR1, 2.0-fold change). As shown in Fig. 4A,B and C, the antioxidant protein 1 homolog gene (ATOX1) was up-regulated after H_2O_2 treatment (2.7-fold change) and was highly up-regulated after extract recovery treatment at 200 and 400 $\mu\text{g ml}^{-1}$ (6.1 and 10.2-fold change, respectively). The small inducible cytokine subfamily A5 gene (CCL5) was

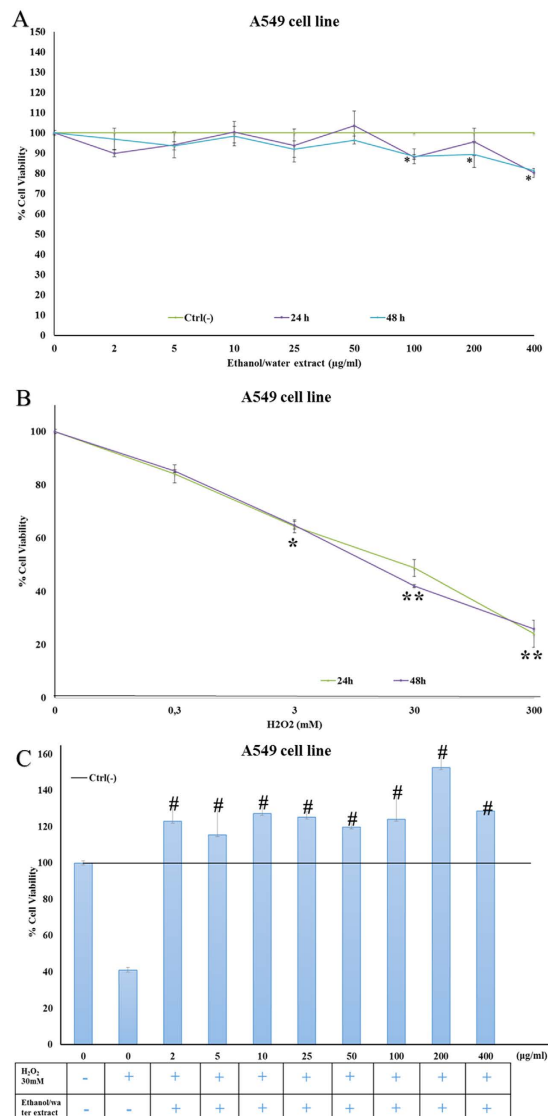


Figure 3. *In vitro* repairing activity of *Tetraselmis suecica* ethanol/water extract against H₂O₂ treatment. (A) Human lung adenocarcinoma cells (A549) treated with various concentrations of *T. suecica* extracts for 24 and 48 h. Cell viability was determined using the MTT assay and expressed as the percentage of control growing cells. (B) Cell viability of lung adenocarcinoma cells (A549) treated with various concentrations of H₂O₂ (0.3, 3, 30, 300 mM) for 24 and 48 h. (C) Effect of extract on cell viability of A549 cells following exposure to H₂O₂ prior to extract treatment at 2, 5, 10, 25, 50, 100, 200 and 400 µg ml⁻¹. Three independent assays were performed in triplicate; data are shown as mean ± S.D. Significant differences between treated groups were determined using Student's-t test (*) and ANOVA followed by Dunnett's test (#). Cross-hatched symbols denote significant differences between treatments and control (*p < 0.05).

down-regulated with H₂O₂ (4.3-fold change) and was up-regulated with the extract at all concentrations tested (2.7, 4.3 and 8.9-fold change, respectively). Opposing gene expression patterns between H₂O₂ and extract recovery treatment at all concentrations tested were recorded in: 24-dehydrocholesterol reductase (DHCR24, -2.9 vs 2.5, 4.8 and 13.2-fold change), forkhead box M1 (FOXM1, -5.1 vs 2.5, 4.5 and 6.0-fold change), glutathione peroxidase 1 (GPX1 -2.0 vs 1.2, 2.0 and 2.1-fold change) and glutathione peroxidase 4 (GPX4, -1.04 vs 1.0, 2.5 and 2.4-fold change, respectively), glutathione S-transferase pi 1 (GSTP1, -4.9 vs -1.1, 3.6 and 3.4-fold change), C-terminal lim domain protein 1 (PDLIM1, -2.11 vs 2.8, 2.2 and 4.3-fold change) and aldo-keto reductase family 1, member C2 (AKR1C2, -2.6 vs 1.1, 2.03 and 10.0-fold change). On the contrary, peroxiredoxin 5 (PRDX5), solute carrier family 7 (SLC7A11) and heat shock protein alpha class A member 1 (HSP90AA1, 90 kDa) were up-regulated with H₂O₂ (2.2, 6.2 and 2.3-fold change) and down-regulated or poorly expressed at 100 µg ml⁻¹ (1.7, -1.6 and 6.0-fold change), 200 µg ml⁻¹ (-1.6, 7.2 and -1.8-fold change) and 400 µg ml⁻¹ (-6.0, -12.3 and -4.1-fold change). Interestingly, prostaglandin reductase 1 (PTGR1) was down-regulated after H₂O₂ injury (-2.5-fold change) and was even up-regulated after extract treatment at all concentrations (1.8, 2.0 and 9.0-fold change). Gene expression of the sirtuin 2 (SIRT2) and superoxide dismutase (SOD2) was enhanced by extract

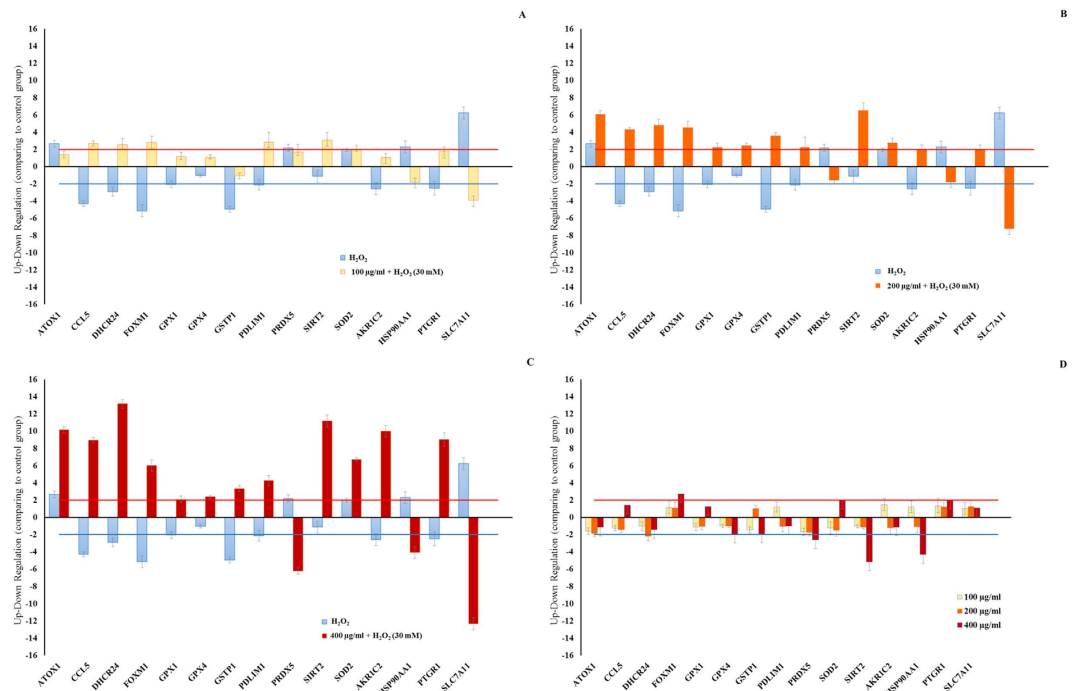


Figure 4. (A,B,C) Effect of *Tetraselmis suecica* ethanol/water extract at three different concentrations (100, 200 and 400 $\mu\text{g ml}^{-1}$) on oxidative stress gene expression in H_2O_2 -treated human lung adenocarcinoma cells (A549). A549 cells were pretreated with H_2O_2 (30 mM = 12 $\mu\text{g ml}^{-1}$) for 1 h prior to extract treatments (100, 200 and 400 $\mu\text{g ml}^{-1}$) and harvested 2 h later. (D) Negative control for the evaluation of the effect of *Tetraselmis suecica* ethanol/water extract at three different concentrations (100, 200 and 400 $\mu\text{g ml}^{-1}$) on oxidative stress gene expression in human lung adenocarcinoma cells (A549). Three independent assays were performed in triplicate and the data are expressed as mean \pm S.D. Expression values greater or lower than a two-fold difference with respect to the controls were considered significant.

treatment after 1 h of exposure to H_2O_2 (from -1.1 to 3.1 , 6.5 and 11.2 , and from 1.9 to 2.0 , 2.8 and 6.7 -fold change, respectively).

Since DHCR24, GPX4 and PTGR1 genes play crucial roles in antioxidant/anti-inflammatory cell signaling pathways, A549 cells were treated with 200 and 400 $\mu\text{g ml}^{-1}$ extract for 24 h in the presence and absence of 30 mM H_2O_2 and protein levels were analyzed by immunoblot. We chose the two most active concentrations used for PCR array analysis in order to compare the upregulation between gene and protein expression. Exposure time was 24 h since protein expression levels were too low before this time. Immunoblot analysis revealed a significant increase only in the expression of DHCR24 at the two concentrations tested (Fig. 5A), whereas the expression of GPX4 and PTGR1 significantly increased only after H_2O_2 pretreatment at 400 $\mu\text{g ml}^{-1}$ (Fig. 5B,C). In light of these results, we hypothesized that the extract was able to repair peroxidative cell damage by reducing the quantity of prostaglandins. Quantitative ELISA test was used to determine prostaglandin E_2 (PGE_2) levels secreted by A549 cells in cell culture medium before and after extract treatment. As shown in Fig. 6A, A549 cells treated with extracts (100, 200 and 400 $\mu\text{g ml}^{-1}$ concentrations) had the same levels of prostaglandin E_2 (29, 30 and 28 $\text{pg } \mu\text{l}^{-1}$, respectively) as the negative control (30 $\text{pg } \mu\text{l}^{-1}$). On the contrary, there was a significant dose-dependent decrease (90, 29 and 28 $\text{pg } \mu\text{l}^{-1}$) in prostaglandin E_2 levels with respect to the positive control (140 $\text{pg } \mu\text{l}^{-1}$) in A549 cells treated with extract (100, 200 and 400 $\mu\text{g ml}^{-1}$) after pretreatment with 30 mM of H_2O_2 (Fig. 6B). Our data show that extract treatment results in a significant decrease in PGE_2 levels in cells damaged by H_2O_2 .

Finally, we used the reconstructed human epidermal tissue model EpiDerm EPI-200 (size 0.63 cm^2) as *in vitro* model to confirm the potential application of this extract as cosmeceutical. In particular, we chose this tissue because *in vivo* oxidative stress frequently occurs in the epidermidis causing aging and other oxidative stress-related diseases. EPI-200 was treated with 30 mM H_2O_2 and with 200 $\mu\text{g ml}^{-1}$ of the ethanol/water extract for 1 h, after injury with H_2O_2 . The epidermal tissue model was treated with 200 $\mu\text{g ml}^{-1}$ of the extract since this was the lowest concentration at which gene and protein expression data revealed a complete activation of all key factors in the antioxidant pathway. Treated medium was removed and replaced with extract, but without H_2O_2 , to assess if the irritant effect persisted after 24 h of recovery (referred to as recovery time). Treatment with 200 $\mu\text{g ml}^{-1}$ extract for 1 h significantly affected tissue viability after 24 h recovery time (85% viability) compared to the irritant effect of 1 h treatment with H_2O_2 (18% viability) (Fig. 7). The repairing effect by the extract was even more evident 1 h later after treatment with 30 mM H_2O_2 and 200 $\mu\text{g ml}^{-1}$ of the extract tissue, with viability increasing from about 20% to 108.5% with respect to the negative control.

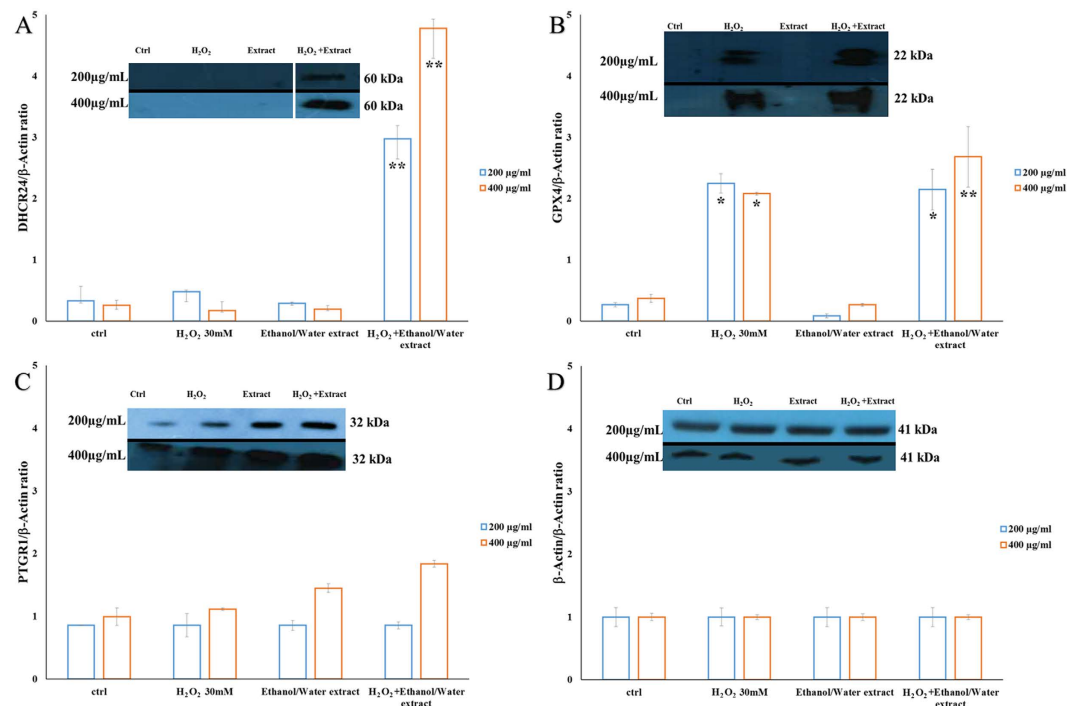


Figure 5. The effect of *Tetraselmis suecica* ethanol/water extract on oxidative stress protein expression in H₂O₂-treated human lung adenocarcinoma cells (A549). Three independent assays were performed in triplicate and the data shown are mean ± S.D. The values above the blots represent the densitometric analysis of the photographic sheets measuring the variation in protein expression. The values of the bands are normalized versus actin and represented as ratio between the expression of single protein and actin. Asterisks denote significant differences compared to controls (*p ≤ 0.05 and **p < 0.005).

Discussion

Previous studies have shown that total extracts of *Tetraselmis* sp. have potential cosmetic and pharmaceutical applications for the human hair growth and pigmentation of the skin, and also for stimulating the increased production of skin structural proteins such as filaggrin and involucrin involved in dermal diseases as Psoriasis⁸. In a previous study, an ethanol/water extract of *Tetraselmis suecica* showed a strong scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and peroxy radicals rather than against the superoxide anions from the xanthine/xanthine oxidase system⁹. Here we show that a similar type of extract from *T. suecica* also stimulates a strong response to cell damage and activates a repairing mechanism in human epidermal cells. Our extract contains high levels of xanthophylls (lutein, violaxanthin, neoxanthin, antheraxanthin and lodoxanthin esters), pigments that are well known for their biological activities as antioxidants and which are precursors of other pigments or vitamins¹⁰. Lutein, which was particularly abundant in this extract, possesses pronounced free radical scavenging ability due to its polarity and number of conjugated double bonds¹¹, and has been shown to significantly decrease neurogenic inflammatory response in the mouse skin¹². The antioxidant activity of violaxanthin and neoxanthin is also well documented¹³, whereas the biological activity of antheraxanthin and lodoxanthin esters recently characterized in *T. suecica*¹⁴ have not yet been investigated.

In our study, the ethanol/water extract, containing high levels of carotenoids, from *T. suecica* showed marked radical scavenging ability when tested with the DPPH assay. The addition of extract led to 98% reduction of the radical DPPH (purple) into its reduced (yellow) form at the highest concentrations. As reported in Table 2, the radical scavenging activity of the extract was dose-dependent and its strength was 70% greater than α-Tocopherol at the highest concentrations. Moreover, the inhibition of DPPH free radicals induced by the extract was comparable to other well-known antioxidant molecules such as ascorbic acid where the percentage reduction of the free radical is about 95%¹⁵.

In order to evaluate this effect at the cellular level we challenged cells with H₂O₂ because a previous study showed that A549 lung cells have a multifaceted response when exposed to hydrogen peroxide¹⁶. D'Andrea *et al.*¹⁶ have shown that H₂O₂ induces damage to lipids, proteins, and nucleic acids due to the generation of reactive oxygen species (ROS). In our case, H₂O₂ caused a reduction in A549 cell viability to 36% after 48 h, but addition of the extract induced a strong recovery in cell viability, with up to 100% recovery in some cases. In order to clarify this effect at the molecular level, we studied the difference in oxidative stress gene expression patterns between cells treated only with 30 mM H₂O₂ and cells recovered with 100, 200 and 400 µg ml⁻¹ extract. The genes involved in ROS metabolism such as oxidative stress responsive genes ATOX1, CCL5 (RANTES), DHCR24, FOXM1, GPX1, GPX4, PDLIM1, PRDX5, SIRT2, SOD2 were all significantly up-regulated in a dose dependent manner after extract recovery treatment for the highest concentrations tested.

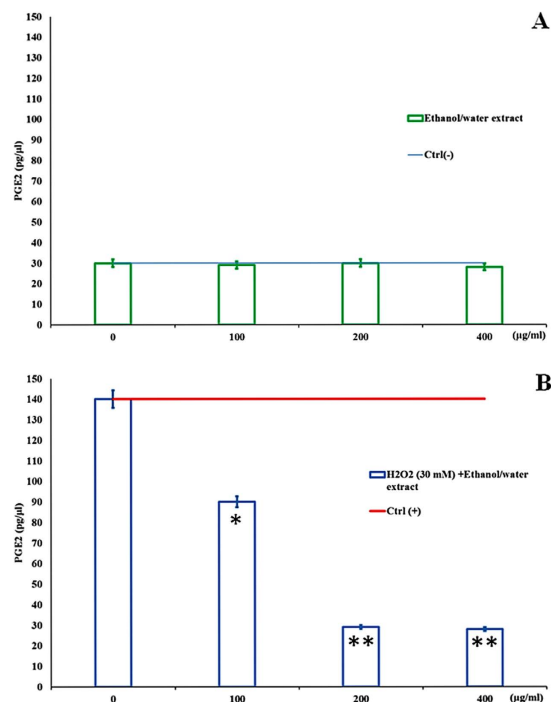


Figure 6. The effect of ethanol/water extract from *Tetraselmis suecica* on prostaglandin PGE₂ serum-release induced by H₂O₂-treatment in human lung adenocarcinoma cells (A549). (A). Average PGE₂ concentration (pg µl⁻¹) determined by ELISA in culture medium of cells treated with 100, 200 and 400 µg ml⁻¹ of the extract for 24 h. (B). Average of the PGE₂ concentration (pg µl⁻¹) determined by ELISA in culture medium of cells treated with 100, 200 and 400 µg ml⁻¹ of extract for 24 h after pretreatment with 30 mM (= 12 µg ml⁻¹) of H₂O₂ for 1 h. Asterisks denote significant differences compared to controls (*p ≤ 0.05 and **p < 0.005) and determined using Students-t test.

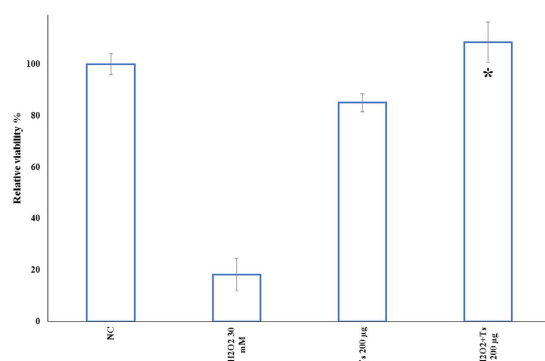


Figure 7. Response of EpiDerm™ tissue cultures after topical application of 30 mM (= 12 µg ml⁻¹) H₂O₂ for 1 h prior to *Tetraselmis suecica* ethanol/water extract treatment (200 µg ml⁻¹) showing the repairing effect of the extract after H₂O₂ treatment. Three independent assays were performed in triplicate; data are shown as mean ± S.D. Significant differences between treated groups were determined using Students-t test (*p ≤ 0.05) and ANOVA. NC = not treated. Ts 200 µg = 200 µg *T. suecica* extract; H₂O₂ + Ts 200 µg = epidermal tissue pretreated with H₂O₂ for 1 h and recovered with extract.

In particular, antioxidant genes such as GPX1 and GPX4 showed a completely reversed expression after extract-induced recovery with respect to H₂O₂ injury for all concentrations tested. On the contrary, PRDX5 showed an up-regulation with H₂O₂, because this gene codes for a mitochondrial peroxiredoxin¹⁷ that decomposes hydrogen peroxide. However, after recovery treatment with extract, PRDX5 gene expression levels decreased in a dose dependent manner and were significantly down-regulated at 400 µg ml⁻¹. This interesting finding is probably due to the ability of the extract to scavenge the effect induced by H₂O₂ thereby reducing PRDX5 gene expression levels.

The genes involved in ROS metabolism such as SOD2 were up-regulated with H₂O₂. Recovery treatment with the extract caused an enhanced up-regulation of this gene in a dose dependent manner. This is an important

finding because, as demonstrated in previous studies, mitochondrial SOD2 plays a crucial role in protecting cells against oxidative stress¹⁸. Another important result regards the effect of the extract on up-regulation of DHCR24 because this is a multifunctional enzyme, which exerts resistance against oxidative stress and prevents apoptotic cell death when it is expressed at high levels¹⁹. The increase in expression levels of the inflammatory pathway gene PTGR1 suggests a potential anti-inflammatory activity because this enzyme is responsible for the biological inactivation of prostaglandins and related eicosanoids²⁰.

The down-regulation of the GSTP1 gene after H₂O₂ treatment indicates that cells were unable to defend themselves against injury. Surprisingly, after 200 and 400 µg ml⁻¹ of the recovery treatment with the extract, GSTP1 was significantly up-regulated indicating the restoration of the antioxidant defense mechanisms.

Peroxide metabolism genes such as AKR1C2, HSP90AA1 and SLC7A11 showed different expression patterns. In particular, the AKR1C2 gene, which catalyzes the conversion of aldehydes and ketones to their corresponding alcohols²¹, was down-regulated with H₂O₂ treatment and up-regulated in a dose-dependent manner by the recovery treatment with the extract (only for 200 and 400 µg ml⁻¹). The activation of this gene indicates a specific cell response to hydrogen peroxide metabolites through the induction of detoxification mechanisms. Heat shock protein 90 kDa alpha (cytosolic) class A member 1 (HSP90AA1) was up-regulated by H₂O₂ treatment because it is a pro-apoptotic factor which induces cell death in response to stress. The extract was able to down-regulate HSP90 expression leading to the induction of cytoprotective pathways through the inhibition of pro-apoptotic pathways²². Surprisingly, the extract was able to down-regulate the SLC7A11 gene which is up-regulated by the H₂O₂ treatment. SLC7A11 encodes a subunit of the xCT cystine/glutamate aminoacid transport system, which is involved in the generation of glutathione and the protection of cells from oxidative stress. However, in a recent study the expression of SLC7A11 was shown to promote tumorigenesis and chemotherapy resistance²³. The down-regulation of SLC7A11 by the extract may therefore be considered as a potential chemo-preventive agent.

In order to demonstrate the activation of an oxidative stress response pathway after *T. suecica* extract recovery treatment, we also analyzed the expression of key proteins involved in antioxidant mechanisms (GPX4 and DHCR24) and PTGR1. Immunoblot data confirmed gene expression results on the induction of an antioxidant pathway in A549 cells damaged with H₂O₂ and then treated with extract (Schematic representation of the oxidative stress response pathway in Supplementary Information, Figure S3). We found that the physiological increase of the active form of GPX4 protein after 30 mM H₂O₂ treatment was enhanced by recovery treatment with 200 and 400 µg ml⁻¹ extract. Another important finding was the increased expression of DHCR24 and PTGR1 proteins induced by extract treatment. The high up-regulation of PTGR1 could be linked to a reduction in prostaglandin release by cells. ELISA experiments showed a significant dose-dependent decrease in prostaglandin PGE₂ levels in the culture medium after recovery treatment confirming this hypothesis. To our knowledge, this is the first report that an ethanol/water extract from a marine green microalga acts as an inhibitor of prostaglandin release in an inflammatory response.

Due to difficulties in obtaining sufficient human lung epithelial cells to perform this study, we chose the A549 cell line derived from a lung carcinoma due to its considerable use in the literature as a surrogate cell type^{24–27} due to its high levels of glutathione²⁸ and high (non-induced) heme oxygenase 1 (HO-1) gene expression levels²⁹. We are aware that results obtained from this transformed cell line may not be applicable and immediately transferrable to normal lung epithelial cells in the respiratory tract *in vivo*. Thus, in order to use this extract as a potential cosmetic agent for topical application, we used human epidermis tissue (EPI-200) as an experimental model and showed that the extract exerted a strong repairing effect after injury caused by hydrogen peroxide.

To date, intervention trials with single antioxidants in pharmacological doses have not supported a repairing effect in humans¹². However, if many antioxidants work in a network, 'total antioxidants' may be a better concept than individual antioxidants. Thus, the potential synergistic effects of bioactive components, such as carotenoids, with different chemical structures and anti-oxidizing activities may be a promising agent for cosmeceutical use. The identification of potent marine microalgal species as peroxide scavengers and repairing agents can lead to new alternative cosmeceutical products or nutritional supplements for prevention of disorders related to oxidative damage, such as cancer, aging and skin inflammation diseases. In order to develop new natural cosmeceutical products for human health applications from marine microalgae, further studies are required to clarify if this bioactivity is ascribable to a single compound, classes of molecules (e. g. carotenoids) or the synergistic effect of several molecules contained in the ethanol/water extract of *Tetraselmis suecica*.

Methods

Strain and culture conditions. The prasinophyte *Tetraselmis suecica* was purchased from the American marine phytoplankton collection (CCMP 906) and was grown in Guillard's *f/2*³⁰ medium without silicic acid in two-liter polycarbonate bottles, constantly bubbled with air filtered through 0.2 µm membrane filters. Cultures were grown at 19 °C, a photon-flux density of about 150 µmol photons m⁻² s⁻¹, and a photoperiod of 12:12 h light:dark (12 L:12 D) cycle. Initial cell concentrations were about 5 × 10³ cells ml⁻¹. Microalgal biomass was collected by centrifugation after 9 days (~8 × 10³ cells ml⁻¹).

Preparation of ethanol/water extract from *Tetraselmis suecica*. Extraction was performed according to Goiris *et al.*³¹. Extraction procedure was conducted under dark conditions, at room temperature and under nitrogen atmosphere, in order to avoid oxidation of the sample. Freeze-dried biomass (~100 mg) was extracted with 1 ml ethanol/water (3/1 v/v) mixture for 30 min. The mixture was separated by centrifugation at 4500 × g, for 10 min, at 20 °C, and the upper layer was transferred to a clean tube. The pellet was resuspended in 1 ml of the ethanol/water mixture and extracted for a second time. The ethanolic extract was dried in a rotary vacuum evaporator (Buchi rotavapor R-114). Dry extract was stored under nitrogen atmosphere at -20 °C prior to analysis.

HPLC analysis of bioactive pigments. Pigment measurements were conducted by High Performance Liquid Chromatography (HPLC) on an aliquot of the ethanolic extract (10 mg), according to methods described in³². Prior to injection into the HPLC, 250 μ L of an Ion Pairing Agent (ammonium acetate 1 mol L⁻¹, final concentration of 0.33 mol L⁻¹) was added to 0.5 mL of the pigment extract and incubated for 5 minutes in the dark at 4 °C. This extract was then injected into the 50 μ L loop of the Hewlett Packard series 1100 HPLC (Hewlett Packard, Wilmington, NC, USA), equipped with a reversed-phase column (C8 Kinetex column; 50 mm \times 4.6 mm; 2.6 μ m particle size, Phenomenex[®], USA). The temperature of the column was steadily maintained at 20 °C, and the flow rate of the mobile phase was set up at 1.7 mL min⁻¹. The mobile phase was composed of two solvent mixtures: A, methanol/aqueous ammonium acetate (70/30, v/v) and B, methanol. During the 12-minutes elution, the gradient between the solvents was programmed: 75% A (0 min), 50% A (1 min), 0% A (8 min) isocratic for 3 min. Chlorophylls and carotenoids were detected by diode-array spectroscopy (spectrum data collected in the range 350–750 nm) using a Hewlett Packard photodiode array detector, model DAD series 1100 and absorbance chromatogram was reported at 440 nm. Chlorophylls were also detected by fluorescence using a Hewlett Packard standard FLD cell series 1100 with excitation and emission wavelengths set at 407 nm and 665 nm, respectively. Identification and quantification of pigments were carried out using pigment standards from the D.H.I. Water & Environment (Horsholm, Denmark). Pigment standards derived primarily from phytoplankton. The standards are flushed with 100% N₂ and supplied in sealed vials with 2.5 mL, together with a certificate of analysis. All information regarding the accuracy of the preparation of the standards and reference numbers of all standards are available at the following address: <http://c14.dhigroup.com/productdescriptions/phytoplanktonpigment-standards>. Moreover spectral information was compared with a library of chlorophyll and carotenoid spectra of pigments prepared from standard phytoplankton cultures³³.

LC-MS/MS analysis of bioactive pigments. LC-MS/MS analysis was carried out on a Waters Alliance HPLC with a Waters 996 PDA detector on line with a Q-ToF mass spectrometer (Waters) featured by an ESI source in positive ionization mode. Column: Phenomenex Luna C8 250 \times 4.6 mm, 5 μ m, 100 A. Eluent A: Water, B: MeOH. Gradient: 90% B to 100% B in 15 mins, holding for 20 mins. Flow: 0.7 ml min⁻¹. PDA: 400–700 nm. Full Mass range: 450–1000 *m/z*.

Scavenging activity against DPPH radical. 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) was used for the radical scavenger assay (Sigma Aldrich, cat. 257621). Various concentrations of extract from *T. suecica* were mixed with a final concentration of DPPH of 0.1 mM in methanol, allowed to react for 30 min in the dark, and absorbance was measured at 517 nm using a microplate reader.

Treatment of Human Cells. The adenocarcinomic human alveolar basal epithelial cell line A549 was purchased from the American Type Culture Collection (ATCC[®] CCL185TM) and grown in DMEM-F12 (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum (FBS), 100 units ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin in a 5% CO₂ atmosphere at 37 °C. A549 cells (2 \times 10³ cells well⁻¹) were seeded in a 96-well plate and kept overnight for attachment. The extract was dissolved in dimethyl sulfoxide (DMSO) and used for the treatment of cells. Seventy percent confluent cells were treated with extract at 2, 5, 10, 25, 50, 100, 200 and 400 μ g ml⁻¹ for 24 and 48 h in complete cell medium. The final concentration of DMSO used was 1% (v/v) for each treatment.

Cell Viability. The effect of ethanol/water extract on cell viability was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay (Appllichem A2231) in according to³⁴. A549 cells, seeded in 96-well plates (2 \times 10³ cells/well), after treatment times, were treated with 10 μ l (5 mg ml⁻¹) of MTT and incubated for 3 h. The absorbance was recorded on a microplate reader at a wavelength of 570 nm (Multiskan FC, THERMO SCIENTIFIC). The effect of the extract on cell viability was evaluated as percent of cell viability calculated as the ratio between mean absorbance of each sample and mean absorbance of control.

RNA Extraction and Real-Time PCR. A549 cells (2 \times 10⁶), used for RNA extraction and analysis, were seeded in Petri dishes (100 mm diameter) to obtain four types of samples: negative control without any treatment, positive control with 30 mM of H₂O₂, cells treated with ethanol/water extracts (100, 200 and 400 μ g ml⁻¹), and cells recovered with extracts (100, 200 and 400 μ g ml⁻¹) after pre-treatment for 1 h with 30 mM H₂O₂. After 2 h of exposure time, A549 cells were washed directly in the Petri dish by adding cold Phosphate-Buffered Saline (PBS) and rocking gently.

Cells were lysed in the Petri dish by adding 1 ml of Trisure Reagent (Bioline, cat. BIO-38033) per 100 mm dish diameter. RNA was isolated according to the manufacturer's protocol. RNA concentration and purity was assessed using the nanophotometer Nanodrop (Euroclone).

About 200 ng RNA was subjected to reverse transcription reaction using the RT² first strand kit (Qiagen, cat.330401) according to the manufacturer's instructions. The qRT-PCR analysis was performed in triplicate using the RT² Profiler PCR Array kit (Qiagen, cat.330231), in order to analyze the expression of cell oxidative stress genes on A549 cells. Plates were run on a ViiA7 (Applied Biosystems 384 well blocks), Standard Fast PCR Cycling protocol with 10 μ l reaction volumes. Cycling conditions used were: 1 cycle initiation at 95.0 °C for 10 min followed by amplification for 40 cycles at 95.0 °C for 15 s and 60.0 °C for 1 min. Amplification data were collected via ViiA 7 RUO Software (Applied Biosystems). The cycle threshold (Ct)-values were analyzed with PCR array data analysis online software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>, Qiagen).

Protein Extraction and Western Blotting. A549 cells (2 \times 10⁶), used for protein extraction and analysis, were seeded in Petri dishes (100 mm diameter) to obtain four types of samples: negative control without any treatment, positive control with cells treated with 30 mM H₂O₂, cells treated with 100, 200 and 400 μ g ml⁻¹, and cells recovered with 100, 200 and 400 μ g ml⁻¹ of extract after pre-treatment for 1 h with 30 mM H₂O₂. A549 cell

lysate was prepared after 24 h of treatment by scraping the cells of each Petri dish into 1 ml of Radio Immune Precipitation Assay buffer (RIPA, Cell Signaling, cat. 9806), supplemented with 1 μ M of protease inhibitor PMSF (Cell Signaling, cat. 8553). The lysate was incubated on ice for 15 min and then clarified by centrifugation at 14000 \times g, for 20 min. Total protein concentration was determined according to the Bradford method using a Protein Assay Reagent (Applichem, cat. A6932) with bovine serum albumin (BSA, Sigma Aldrich, cat. A2058) as a standard. The protein extract was stored at -20°C until use. Before electrophoresis, protein samples were incubated at 100°C for 5 min. Following 10% SDS-PAGE, gels were stained with Coomassie or blotted onto nitrocellulose membrane (Biorad, cat. 170–4159). Membranes were incubated for 1 h in blocking reagent (1X Tris Buffered Saline-TBS), with 0.1% Tween-20 with 5% w/v nonfat dry milk, and incubated overnight at 4°C with the primary antibodies diluted in 1X TBS, 0.1% Tween-20 with 5% BSA.

Three key proteins were investigated: 24-dehydrocholesterol reductase (DHCR24, 1:1000, Sigma Aldrich SAB1405713), glutathione peroxidase 4 (GPX4, 1:1000, Sigma Aldrich SAB2500486), prostaglandin reductase 1 (PTGR1, 1:1000, Sigma Aldrich SAB4500918). Positive control was obtained by using anti- β -actin antibody (1:500, Novus Biological cat. NB600-501).

After incubation, membranes were washed three times for 5 min each with 15 ml of TBS/Tween and then incubated with HRP-conjugated secondary antibody with gentle agitation for 1 h at room temperature. For β -actin and DHCR24 antibodies, we used HRP-conjugated secondary antibody anti-mouse (1:10000, Santa Cruz Biotechnology); for GPX4 and PTGR1 antibodies, we used HRP-conjugated secondary antibody anti-rabbit (1:10000, Jackson ImmunoResearch).

After incubation, membranes were washed three times for 5 min each with 15 ml of TBS/Tween. Blotted membranes were immunodetected using clarity Western ECL (Biorad, cat. 170–5060). Proteins were visualized with Fuji medical X-ray film (cat. 47410). Densitometric analysis of immunopositive bands was performed using Image J software.

ELISA for PGE2. Prostaglandins were quantified in the cell medium by ELISA kit (Life Technologies, cat. EHPGE2) according to standard manufacturer's recommendations. We quantified prostaglandins in four samples: A549 cells without treatment (control), A549 cells treated with 100, 200 and 400 $\mu\text{g ml}^{-1}$ of extract, A549 cells treated with only 30 mM of H_2O_2 and A549 cells treated with 100, 200 and 400 $\mu\text{g ml}^{-1}$ of the extract after pre-treatment for 1 h with 30 mM H_2O_2 .

Treatment of human epidermis. EpiDermTM was purchased from the MatTek Corporation (EPI-200-SIT) and maintained in culture medium provided by the manufacturer and according to a standardized protocol. The EpiDerm skin model (area of 0.6 cm^2) consisted of normal human derived epidermal keratinocytes (NHEK) cultured to form a multilayered, highly differentiated model of the human epidermis.

EpiDerm tissues were conditioned by incubation with culture medium in order to release transport-stress related compounds and debris overnight (in a 5% CO_2 atmosphere at 37°C). About 200 $\mu\text{g ml}^{-1}$ carotenoid extract was dissolved in dimethyl sulfoxide (DMSO, final concentration 1% v/v) and used for the treatment of human epidermis; after 1 h of incubation with the extract, a complete recovery medium was used for 24 h of recovery time.

Tissue Viability. The effect of the extract on tissue viability was determined using the MTT assay. After 1 h treatment and 24 h of recovery time, tissues were transferred to 24-well plates containing MTT medium (1 mg ml^{-1}). After 3 h incubation in MTT, the blue formazan salt was extracted with 2 ml/tissue of isopropanol and the optical density of the extracted formazan was determined using a spectrophotometer at a wavelength of 570 nm. Relative tissue viability was calculated for each tissue as percentage of the mean of the negative control tissues. Skin irritation potential of the test extract was predicted if the remaining relative tissue viability was below 50% (MatTek Corporation-Protocol).

Statistical Analysis. Statistical significance of the DPPH assay was determined by Students-t test (*p values ≤ 0.05). Statistical differences between treated and control cells for cell viability counts were determined by One-way ANOVA and significant differences between the treated groups by Students-t test (*) and ANOVA followed by Dunnett's test (#) (p values ≤ 0.001) using Microsoft Excel software (365 version, 2013). Gene expression data were analyzed by PCR array data analysis online software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>, Qiagen[®]). Only expression values greater than a 2.0-fold difference with respect to the controls were considered significant. Immunoblotting protein expression was calculated as the percentage of integral area of every single gel band with respect to total gel lane area, represented as pixels. Statistical differences between treated and controls were determined by Students-t test with significant p values ≤ 0.05 . Data significantly different from controls, with p values < 0.001 are marked with two asterisks in the figures. Significant differences between treated groups, after epidermal human tissue experiments, were determined using Students-t test (*p ≤ 0.05) and ANOVA.

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Author Contributions

Conceived and designed the experiments: A.I., G.R., C.S. and C.G., C.S. and C.G. took part and contributed equally in the experiments with cell culture, real-time-PCR and western blotting. G.N., E.L. and A.F. performed the chemical extractions. A.C. performed LC-MS/MS analysis. I.O. and C.B. performed HPLC analysis. F.E. cultured the microalga. Analyzed the data: A.I., G.R., C.B., A.F., C.S., C.G. Contributed reagents/materials/analysis tools: AI GR. Wrote the paper: A.I., G.R., C.S. and C.G.

Additional Information

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