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The Cosmeceutical Potential of the Yellow-Green Alga *Trachydiscus minutus* Aqueous Extract: Preparation of a Natural-Based Dermal Formula as a Proof of Concept

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Abstract: In the present study, selected cosmeceutical properties of aqueous extracts from the microalgae strain *Trachydiscus minutus* were assessed and compared with those obtained using three widely used *Chlorella* strains (*C. vulgaris*, *C. sorokiniana*, and *C. minutissima*). Among all extracts, *T. minutus* extracts showed the highest total antioxidant capacity (TAC) and inhibitory potency towards elastase, suggesting potential activity in controlling skin aging. Furthermore, the cytotoxicity, anti-inflammatory activity and UVA protection of *T. minutus* extract were evaluated employing normal human dermal fibroblasts (NHDF) and human keratinocyte HaCaT cells. The results showed that the *T. minutus* extract was able to significantly inhibit the transcription of selected marker genes involved in inflammation [interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor alpha (TNFα)]. In addition, treatment of NHDF and HaCaT cells with *T. minutus* extract ameliorate the UVA-induced cell damage by decreasing the accumulation of reactive oxygen species (ROS). Extracts from *T. minutus* were formulated into a skin care cream and an aqueous gel. Both formulas exhibited excellent compatibility and stability. Comprehensively, all these results suggest that *T. minutus* extract displays promising cosmeceutical properties by providing antioxidant, anti-aging, and anti-inflammatory activities, and therefore has potential for cosmeceutical use.

Keywords: anti-aging; antioxidant; cosmeceutical; elastase; yellow-green microalgae

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1. Introduction

Microalgae produce an extraordinary variety of often unique active secondary metabolites, closely linked to the unique features of their environmental habitats, consisting of continuous variations in light, pressure, nutrient, salinity, and temperature [1,2]. Since microalgae are very diverse and adaptable to a broad variety of environmental conditions, there is significant opportunity for the detection of novel and unexplored bioactive metabolites with properties suitable for biomedical applications [3–7]. The bioactive metabolites that microalgae produce in order to tolerate stress conditions, such as high or low temperature, photo-oxidation and ultraviolet radiation, reinforce their use in cosmetic industries for the production of anti-aging, sunscreen, and skin whitening products [7–11]. Although the applications of microalgae in cosmetic products have recently received significant attention in the treatment of the skin [12–18], the vast majority of studies are restricted to the use of *Chlorella* or *Arthrospira* strains [4–7,19]. Microalgae diversity, as an asset in the cosmetics

industry, remains largely untapped due to an apparent lack of studies for assessing the bioactivity and utility of new microalgae species as primary active ingredients [11].

Xanthophyceae is a group of photosynthetic organisms found in fresh water, marine waters, and moist soils [20]. Their specific yellow-green colour is attributed to the pigments chlorophyll c, chlorophyll a and β-carotene, although they completely lack the brown pigment fucoxanthin [21,22]. The Xanthophyta were first described in the 1950s [23], and include more than 600 species that have been reported in diverse habitats (e.g., temperate zones of all continents). Among *Xanthophyceae* group, the freshwater eustigmatophyte alga *Trachydiscus minutus* still remains a rather unknown microalga. *Trachydiscus minutus* is considered a valuable source of bioactive metabolites, including eicosapentaenoic acid (EPA), antioxidants, pigments, and structured triacylglycerols [24–29]. Despite its potential, there are limited published data concerning its physiology, biochemistry, and molecular biology [27–29].

Considering the market demand for environmentally friendly and safe cosmetics [1,2,4,7], there is an intense interest in the exploitation of new or underused microalgae species [8–11]. Taking into account the scarcity of the available experimental studies for yellow-green algae and their promising but still unexploited biotechnological potential [24–29], this study aimed to investigate the cosmeceutical potential of *T. minutus*. The results indicated that *T. minutus* extract displays high potential as raw material for the development of cosmeceuticals with desirable properties. To the best of our knowledge, this is the first attempt to integrate bioactivity assessment and application of *T. minutus* extracts as cosmeceuticals.

2. Materials and Methods

2.1. Materials

Enzymes and substrates were obtained from Sigma-Aldrich, MO, USA. Cell lines were from Lonza (Basel, Switzerland). Cell media, supplements, and cell culture reagents were from Gibco (Thermo Fisher, MA, USA). ATP was measured using the ViaLight plus kit (Lonza, Basel, Switzerland). The algae strains used in the present study (*Trachydiscus minutus*, *Chlorella vulgaris*, *Chlorella sorokiniana*, and *Chlorella minutissima*) were a much appreciated gift from Ecoduna (Bruck an der Leitha, Austria). DMEM with high glucose medium (Gibco), L-Glutamine (Sigma-Aldrich), FBS (Sigma-Aldrich), Penicillin/Streptomycin (Life Technologies, CA, USA), Chelex (Sigma-Aldrich), Phosphate Buffered Saline (Sigma-Aldrich), Trypan Blue Solution (Bio-Rad, CA, USA), Trypsin (Sigma-Aldrich), Ethanol from Sigma-Aldrich (Madrid, Spain), ROS detection kit (Sigma-Aldrich), Methanol (Sigma-Aldrich), Chloroform (Sigma-Aldrich), NaCl (Sigma-Aldrich), DNAse-I (Qiagen), RNeasy extraction kit (Qiagen, Germany), PrimeScript RT reagent kit (Perfect Real Time, Takara-Clontech, Japan), and oligonucleotides for RT-PCR amplification of TNFα, IL8, IL-6 and ACT, SYBR®, RT-qPCR, LPS (Sigma-Aldrich) were all obtained. Alamar Blue cell viability reagent [Resazurin sodium salt (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt)] (Sigma-Aldrich), Polyacrylate Crosspolymer-6 (Seppic, France), Sodium Gluconate (Jungbunzlauer International, Switzerland), Cetearyl Alcohol (Basf, Germany), Sodium Cetearyl Sulfate (Basf), Caprylic/Capric Tryglyceride (Croda, UK), Phenoxyethanol (Galaxy Surfactants, India) were also obtained.

2.2. Methods

2.2.1. Algae Extraction

Microalgae cells (1 g lyophilised material) were suspended in suitable buffer (KH_2PO_4, 20 mM, pH 7 or ddH_2O/glycerol 50/50 v/v, 7 mL). Cells were disrupted by sonication (50 Watt, 60 Hz, 5 cycles of 25 s sonication and 60 s intervals in ice bath). Following sonication, the cell lysate was centrifuged at 16,000× g for 10 min, and the resulting supernatant was used for the analysis.

2.2.2. Total Antioxidant Capacity Assays

Ferric Reducing Antioxidant Power (FRAP) assays were performed according to Benzie and Strain [30]. The results were expressed as µg ascorbic acid/mg of algae extract dry mass. 2,2-Diphenyl-1-picrylhydrazine (DPPH) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free-radical scavenger assays are based on the spectrophotometrically determined quenching of the coloured radicals DPPH and ABTS upon their reaction with antioxidants. The assays were carried out according to Carballo et al., 2018 [31]. The DPPH and ABTS activities were expressed as (%) free radical scavenging and the IC50 values (the dry mass of algae required to scavenge 50% DPPH and ABTS radicals) were also estimated by nonlinear or linear regression analysis employing the program GraphPad Prism version 8. The ORAC assay was carried out according to Gillespie et al., 2007, using fluorescein as the probe [32]. ORAC values were calculated by subtracting the area under the blank curve from the area under the sample curve (Net AUC) and expressed as Trolox equivalents (µmol Trolox/mg of dry mass).

2.2.3. Enzyme Inhibition Assays

Elastase was assayed spectrophotometrically using succinyl-Ala-Ala-Pro-p-nitroanilide as substrate. Enzyme activity was measured by monitoring the release of p-nitroaniline at pH 8.0 and 25 °C. The amount of p-nitroaniline was determined at 410 nm [33]. One unit of elastase will hydrolyse 1.0 µmole of substrate per minute at pH 8.0 and 25 °C. The inhibition of elastase (% *I*) was calculated using the following equation:

$$\%Inhibition = \frac{R_0 - R_i}{R_0} \quad (1)$$

where R_0 is the rate produced by the reaction in the absence of an inhibitor and R_i is the rate of the reaction with an inhibitor (extract). Both R_i and R_0 correspond to the same substrate concentrations. All determinations and assays were carried out in triplicate and at least three replications of each experiment were performed. The results have been expressed as the mean values ± SD.

2.2.4. Toxicity Assessment of *T. minutus* Extract on NHDF and HaCaT Cells

The viability of NHDF and HaCaT cells was assessed following treatment with *T. minutus* extract for 24 h. Cytotoxicity was determined after exposure of the cultures to 0–0.02% (*v*/*v*) extract. The respective cell-culture medium without *T. minutus* extract was used as a control. In order to determine live cell count, cell viability was assessed by staining with Trypan Blue solution. Live cells were counted in a Bürker chamber under the microscope. For the Alamar Blue assay, NHDF and HaCaT cells were cultured overnight at a 10,000 cells/well density in a 96-well plate in growth media supplied with 20% and 10% FBS, respectively. After 24 h incubation, the medium was removed, wells were washed with PBS buffer to eliminate any residual medium, and Alamar Blue cell viability reagent (1:10) was added to each well. Plates were incubated at 37 °C for 3 h and the fluorescence was measured at Ex 560/Em 590 nm. Fluorescence values lower than those of control cells indicated a reduction in the rate of cell proliferation. Conversely, a higher fluorescence rate indicated an increase in cell viability and proliferation. A biological replicate with 8 technical replicates per concentration and 16 technical replicates for the untreated control were used. All data were statistically analysed using one-way ANOVA test. Statistical significance was set at $p < 0.05$, 95% of confidence.

2.2.5. Inflammation Assessment of *T. minutus* Extract on NHDF and HaCaT Cells

NHDF and HaCaT cells were cultured at a 200,000 cells/well density in a 6 well plate, in growth medium. After 24 h, the medium was removed and the microalgae extracts were added at two different concentrations (0.002% and 0.001%, *v*/*v*) to the cells. Twenty four hours later, LPS at a final concentration of 100 ng/mL was added to induce an inflammatory response. After 20 h of incubation, cells were washed with PBS buffer and collected in lysis

buffer to proceed with RNA extraction. Total RNA was extracted using RNeasy kit (Qiagen) and treated with DNAse-I to remove any contamination from genomic DNA. RNA quality and quantity were checked in a Nano-Drop spectrophotometer, and 1 µg of total RNA was used to synthesize cDNA, using First-strand Synthesis kit (Takara-Clontech). The primer sequences used for RT-qPCR, (TNFα, IL-6, IL-8 and ACT) are listed in Supplementary Table S1. Quantitative PCR (qPCR) was performed in a real time PCR machine (QuantStudio 5, Applied BioSystems). Raw data analysis was performed using the Pfaffl method to calculate the gene relative expression ratio to ACT (internal control-housekeeping gene) [34]. The mathematical model of the relative expression ratio in real-time PCR is shown below:

$$Ratio = \frac{\left(E_{target}\right)^{\Delta CP_{target}(control-sample)}}{\left(E_{ref}\right)^{\Delta CP_{ref}(control-sample)}} \quad (2)$$

where E_{target} is the real-time PCR efficiency of target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCP_{target} is the CP deviation control − sample of the target gene transcript; and ΔCP_{ref} = CP deviation of control − sample of reference gene transcript. Statistical analysis was performed using ANOVA and Bonferroni's multiple comparison test as a post hoc and unpaired Student's t-test. For all data, a level of 5% or less ($p < 0.05$) was taken as statistically significant.

2.2.6. UVA Protection of *T. minutus* Extract on ROS Accumulation in NHDF and HaCaT Cells

NHDF and HaCaT keratinocytes cells were treated for 24 h with *T. minutus* extract at different concentrations (0–0.02%, v/v), previously selected from cytotoxicity analysis. NHDF and HaCaT cells were cultured overnight at a 10,000 cells/well of density in a 96 well black plate in growth media. After 24 h of incubation, cells were washed twice with PBS, and ROS master mix (Sigma-Aldrich) was added to all cultured wells, immediately before the UVA irradiation. Five control wells without cells were included as a blank control. Cells were exposed to 7.86 J/cm^2 UVA irradiation. Six technical replicates per condition were assessed for ROS accumulation after exposure to UVA light (Potency = 4.3 mW/cm^2, 30 min, total energy = 7.85 J/cm^2). Non-irradiated controls were incubated at 37 °C during this time in the dark. Two hours after ROS master mix addition, fluorescence was measured at λ_{ex} = 490/λ_{em} = 525 nm in all samples, and ROS accumulation was obtained. The intracellular ROS accumulated reacted with a fluorogenic dye, resulting in a fluorogenic product in amounts proportional to the amount of ROS present. Fluorescence data collected from the assay were analysed. Briefly, the mean of background fluorescence (irradiated or not) was subtracted from each measurement in each replicate. The mean values for the replicates in the control (untreated, non-irradiated) and the Control + UVA (untreated, irradiated) were then obtained, and these values were used to normalize each replicate measurement of fluorescence in the corresponding samples and conditions. The tests applied for the analysis were the unpaired Student t-test and ordinary one-way ANOVA. Statistical significance was set at $p < 0.05$, 95% of confidence.

2.2.7. Preparation of Oil in Water (O/W) Emulsion Cream and Aqueous Gel Enriched with *T. minutus* Extract

An oil in water (O/W) emulsion cream and an aqueous gel (face serum) were carried out using 0.1% and 0.5% (w/w) of *T. minutus* extracts (glycerol-aqueous extracts, 50/50 v/v). Initially, the O/W emulsion was prepared through separate heating of the oily phase (cetearyl alcohol, sodium cetearyl sulfate, caprylic/capric triglyceride) and the aqueous phase (water, glycerine, polyacrylate crosspolymer-6, sodium gluconate) at 80 °C (Table 1). The oily phase was subsequently stirred into the aqueous phase followed by intensive agitation with a Silverson mixer. The mixture was maintained under constant stirring until its temperature reached 35 °C. The final emulsion resulted from the addition of phenoxyethanol (1% w/w) and the microalgal extracts (0.1% and 0.5%, w/w).

Table 1. Composition of the water and oil phases used for the preparation of cosmetic cream formulation.

Water Phase	**% (*w*/*w*)**
Water (ddH_2O)	Q.S.
Glycerol	2.0
Polyacrylate Crosspolymer-6	0.5
Sodium Gluconate	0.1
Oil Phase	**% (*w*/*w*)**
Cetearyl Alcohol, Sodium Cetearyl Sulfate, Disodium Phosphate, Potassium Phosphate	5.0
Caprylic/Capric Tryglyceride	10.0
Phenoxyethanol	1.0
[1] *T. minutus* extract (glycerol-aqueous extract, 50/50 *v*/*v*)	% (*w*/*w*)

[1] 0.1% (*w*/*w*) or 0.5% (*w*/*w*) were added in the final preparation.

The aqueous gel formulation was performed with the polyacrylate crosspolymer-6 that was dispersed in the aqueous phase (water, glycerine, and sodium gluconate) at room temperature with intensive agitation using a Silverson mixer (Table 2). The active ingredients (microalgae extract 0.1% and 0.5%, *w*/*w*) along with the preservative were uniformly incorporated into the gel. Both cream and gel were assessed by using the counterpart products without extracts as control measurements.

Table 2. Composition of the water phase used for the preparation of aqueous gel formulation.

Water Phase	**% (*w*/*w*)**
Water (ddH_2O)	Q.S.
Glycerol	2.0
Polyacrylate Crosspolymer-6	1.0
Sodium Gluconate	0.05
Phenoxyethanol	1.0
[1] *T. minutus* extract (glycerol-aqueous extract, 50/50 *v*/*v*)	% (*w*/*w*)

[1] 0.1% (*w*/*w*) or 0.5% (*w*/*w*) were added in the final preparation.

2.2.8. Product Stability

Product stability tests were carried out for the determination of possible physicochemical changes in the samples compared with changes that would be expected in standard storage conditions. Plastic containers, suitable for cosmeceutical use, were filled with approximately 60 g of each sample, which was subsequently exposed to different storage conditions including high and low temperatures (50 °C, 45 °C, 40 °C, 5 °C), room temperature (25 °C), and daylight (UV). A thorough product stability test was typically assessed for three months. All samples under examination were evaluated at the end of each month of storage (0, 30, 60 and 90 days). pH measurements were obtained with a UB-S Denver instrument equipped with a glass electrode, which was inserted directly into the samples. The stability of face cream samples (5 g) was evaluated through mild centrifugation at 3000 rpm for 2 h (two cycles of centrifugation for 1 h interrupted by 30 min interval) and analysis of the eventual separated phases. The evaluation of physical and chemical properties including pH measurements, colour, and phase separation was also applied in formulations that were packaged in appropriate plastic containers.

3. Results and Discussion

3.1. Total Antioxidant Capacity Assays

Total Antioxidant Capacity (TAC) is a significant parameter for the assessment of the antioxidant ability and health beneficial effects of a cosmeceutical [35,36]. This parameter takes into account the synergistic and cumulative interactions between all chemicals present in the sample. In the present work, the TAC of microalgae extracts was evaluated using

the ABTS [37], DPPH [38], FRAP [30,39], and ORAC [40] methods. The results (Figure 1) indicated significant differences in the antioxidant capacity among the selected strains, suggesting a large diversity in antioxidants. Among all tested samples, the *T. minutus* extract displayed the highest antioxidant capacity in all used assays (FRAP, ABTS, DPPH and ORAC).

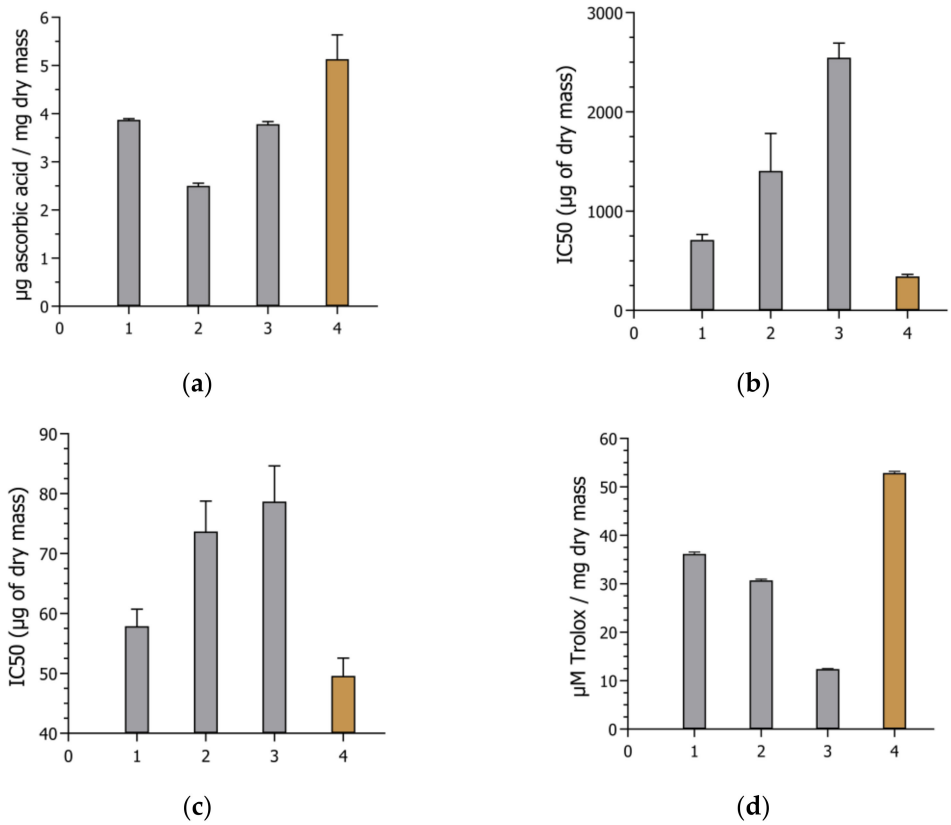


Figure 1. Total Antioxidant capacity of (1) *Chlorella vulgaris*, (2) *Chlorella sorokiniana*, (3) *Chlorella minutissima*, and (4) *Trachydiscus minutus* extracts, expressed as: (**a**) µg of ascorbic acid per mg of dry mass (FRAP); (**b**) IC50 (µg of dry mass), with IC50 being the concentration of an antioxidant-containing substance required to scavenge 50% of the initial DPPH radicals; (**c**) the IC50 (µg of dry mass), with IC50 being the concentration of an antioxidant-containing substance required to scavenge 50% of the initial ABTS radicals; and (**d**) µM equivalent to Trolox per mg of dry mass (ORAC).

3.2. Elastase Inhibition Analysis

The inhibitory effect of the microalgae extracts on the activity of the skin-regulating enzyme elastase was evaluated (Figure 2). The results showed that *T. minutus* extract displayed the higher inhibition potency compared to the *Chlorella* strains. Elastase is capable of breaking down elastin, which, in conjunction with collagen, affects the mechanical properties of connective tissue [35,36]. Several studies have demonstrated that both skin-aging and antiwrinkle effects are significantly correlated with decreased elastase activity [41,42]. In addition, elastase is also involved in various other diseases, such as inflammation, rheumatoid arthritis, cystic fibrosis, chronic obstructive airway disease, psoriasis, and delayed wound healing. Due to the specific role of elastase in the inflammatory process, its inhibition by the *T. minutus* extract encourages its use in cosmetic products for irritated, reactive, and/or senescent epidermis [43].

Taking into account that the *T. minutus* extract displayed the highest antioxidant capacity and inhibition potency against elastase, it was selected for further studies employing in vitro tissue culture experiments with NHDF and HaCaT cells.

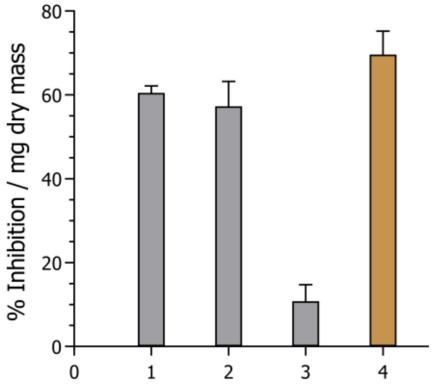
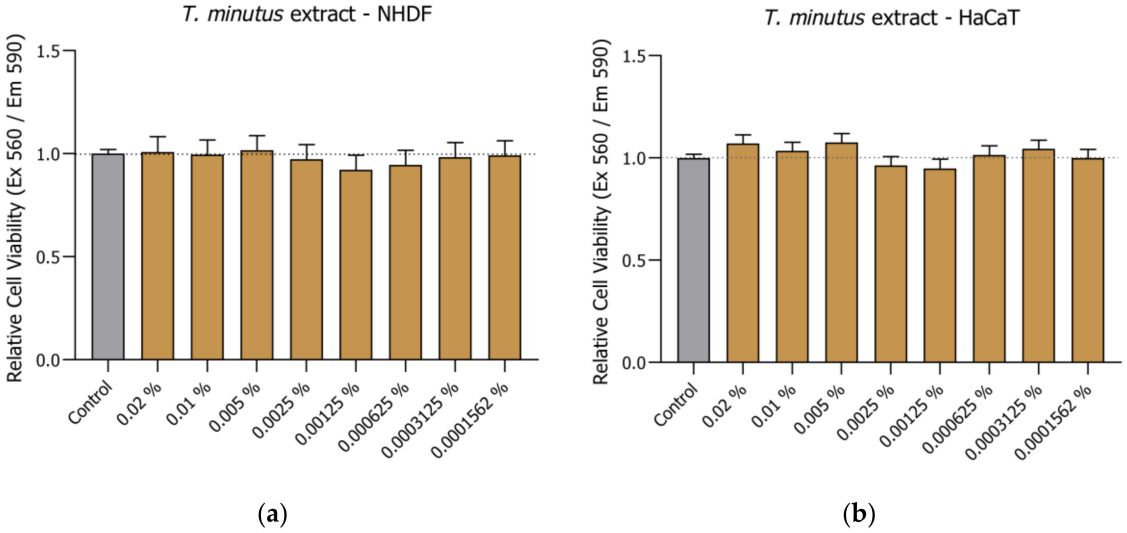


Figure 2. Inhibitory activity (%) of extracts against elastase activity. (1): *Chlorella vulgaris*; (2): *Chlorella sorokiniana*; (3): *Chlorella minutissima*; and (4) *Trachydiscus minutus*.

3.3. Toxicity Assessment of T. minutus Extract on NHDF and HaCaT Cells

Cell viability of NHDF and HaCaT cells was investigated following treatment with *T. minutus* extract for 24 h. The results indicated (Figure 3) that *T. minutus* extracts do not show significant cytotoxicity to NHDF and HaCaT cells after 24 h of treatment, indicating that concentrations up to 0.02% (*v*/*v*) may be used in the subsequent assays, without any relevant decrease in cell viability.



(**a**) (**b**)

Figure 3. Effects of *T. minutus* extract on (**a**) NHDF and (**b**) HaCaT cells viability. NHDF and HaCaT cells were treated with various concentrations of *T. minutus* extract (% *v*/*v*) for 24 h and cell viability was assessed by staining with Trypan Blue.

3.4. Inflammation Assessment of T. minutus Extract on NHDF and HaCaT Cells

The effect of *T. minutus* extract on the transcription of selected cytokine marker genes [interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor alpha (TNFα)], which are involved in inflammation, were evaluated. The inflammatory response on NHDF and HaCaT cells was induced by LPS (100 ng/mL). The biology of different cultured cells when treated with different substances is very complex. This is due to different genetic or epigenetic factors that are expressed. For example, cells exhibit changes in genotype and/or phenotype, possibly as a consequence of external influences. Skin irritation includes different events leading to the development of an inflammatory response at the site of exposure. Cytokines are a family of proteins that regulate the inflammatory and immune response, acting as chemical mediators that are released during the process, and help to intensify and propagate the inflammatory response, often including TNF-α, IL-6 and

IL-8 [44–48]. Although their role in the inflammatory process is complex, TNF-α, IL-6 and IL-8 modulate the activity and function of other cells to coordinate and control the inflammatory response.

The in vitro treatment of HaCaT keratinocytes with LPS, significantly increased the expression of TNFα and IL-8, compared to the untreated control, showing that LPS activated the inflammatory response through these cytokine regulatory pathways (Figure 4a,b). In the case of IL-6, no significant induction was detected after treatment with LPS (Figure 4c). The lack of IL-6 induction at RNA level is probably due to the low concentration of LPS used in the analysis (100 ng/mL), compared to 1 µg/mL that is usually used. When HaCaT keratinocytes cells were previously incubated with *T. minutus* extract for 24 h at 0.002% and 0.01% (*v*/*v*) concentrations, the expression of TNFα (Figure 4a) and IL-6 (Figure 4c) was significantly diminished by 22.5 ± 8.3%, 58.1 ± 7.7% (using 0.002% extract) and 16.1 ± 5.8% and 51.3 ± 5.1% (using 0.01% extract), respectively, compared to the untreated cells in the presence of LPS. On the other hand, the expression IL-8 was unaffected after incubation of keratinocytes with *T. minutus* extract (Figure 4b).

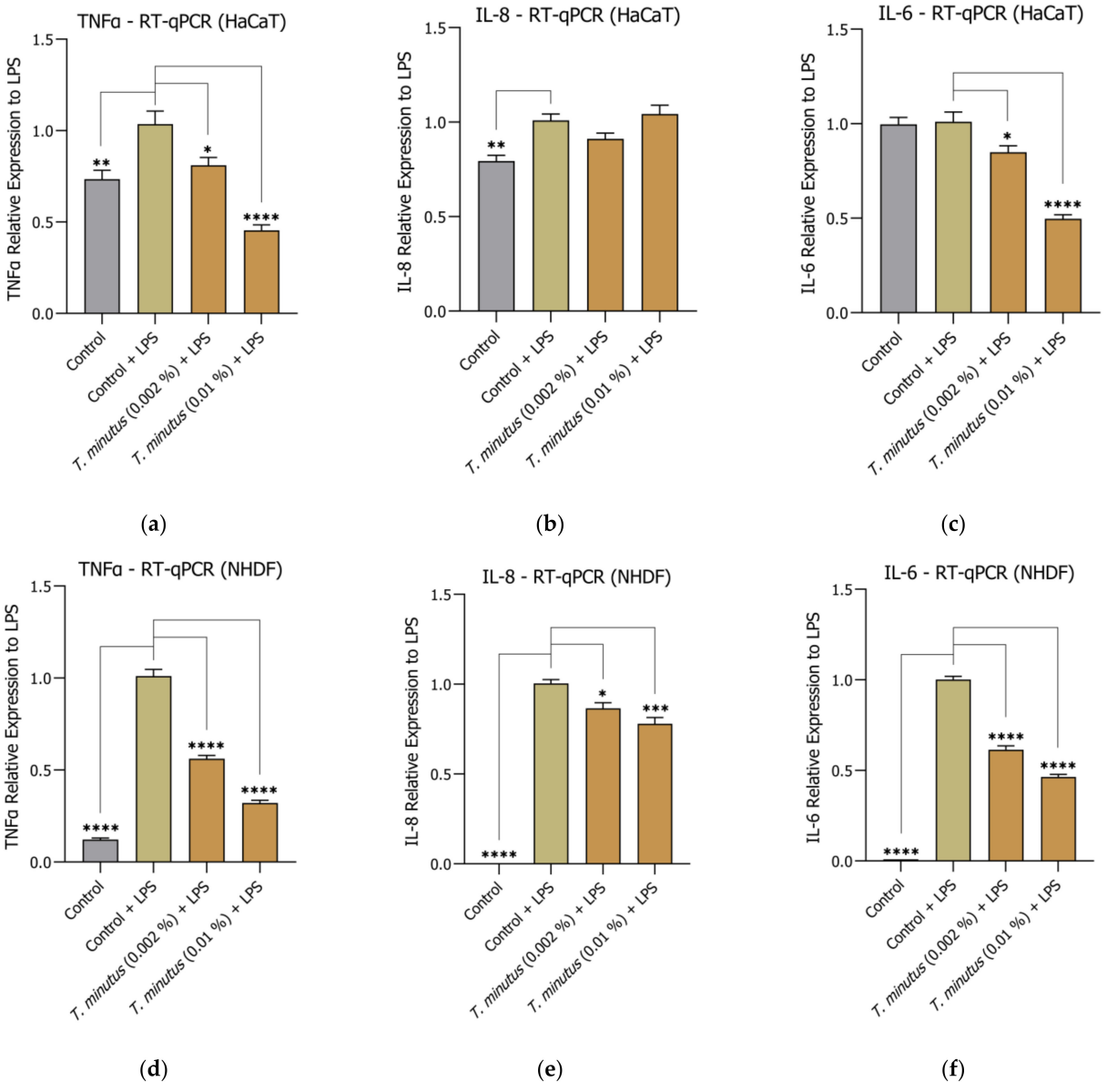


Figure 4. Expression of inflammation-linked marker genes (**a**) TNFα, (**b**) IL-8 and (**c**) IL-6) in LPS-

treated HaCaT cells and (**d**) TNFα, (**e**) IL-8 and (**f**) IL-6) in LPS-treated NHDF cells in the presence or absence of *T. minutus* extract for 24 h. One star (*) indicates statistically significant results ($p < 0.05$), two stars (**) indicate statistically significant results ($p < 0.01$), three stars (***) indicate statistically significant results ($p < 0.001$) and four stars (****) indicate statistically significant results ($p < 0.0001$).

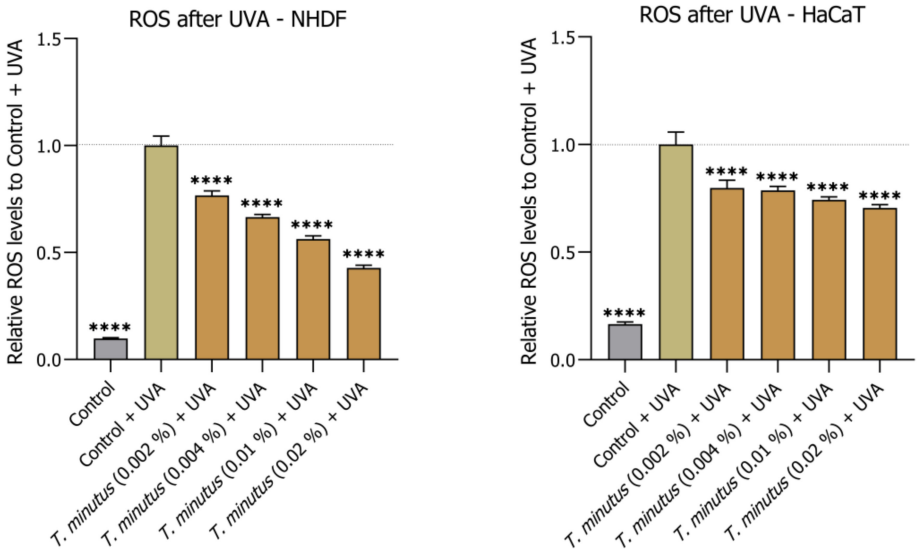
For NHDF, the results indicated that LPS induced the expression of TNFα, IL-6, and IL-8 compared to the untreated control (Figure 4d–f). When cells were previously incubated with 0.002% and 0.01% (v/v) extract, the expression level of TNFα, IL-6, and IL-8 was significantly reduced by 44.8 ± 4.1%, 68.9 ± 4.5%, 38.8 ± 2.8% (using 0.002% extract) and 53.8 ± 2.3%, 13.8 ± 3.9%, and 22.4 ± 3.8% (using 0.002% extract), respectively, compared to the control in the presence of LPS.

The results suggest that in vitro treatment of NHDF cells with *T. minutus* extract for 24 h promotes significant anti-inflammatory effects through inhibition of the LPS-induced TNFα, IL-6 and IL-8 expression on NHDF. Similarly, HaCaT cells showed reduced TNFα and IL-6 expression.

3.5. UVA Protection of T. minutus Extract against ROS Accumulation in NHDF and HaCaT Cells

The antioxidant and protective effect of *T. minutus* extract on HaCaT and NHDF cells, previously subjected to UVA irradiation, was investigated. This study was undertaken in order to evaluate the potential of the extract in counteracting UVA-induced oxidative stress by measuring the accumulation of reactive oxygen species (ROS).

The effect of *T. minutus* extract on ROS accumulation in NHDF and HaCaT cells is illustrated in Figure 5. The results showed that the treatment of HaCaT keratinocytes and NHDF cells with *T. minutus* extract for 24 h provides significant protection against UVA-induced damage by decreasing the accumulation of reactive oxygen species (ROS). UVA irradiation of HaCaT keratinocytes considerably induced the formation of ROS (502.5 ± 14.3%) compared to the untreated control. Previously treated cells with different concentrations of *T. minutus* extract (0.002%, 0.004%, 0.01% and 0.02% v/v) decreased ROS levels by 20.3 ± 4.2%, 21.3 ± 2.7%, 25.6 ± 2.4%, and 29.4 ± 2.5%, respectively. Similarly, for NHDF cells, the results indicated that UVA irradiation increased ROS levels by 921.7 ± 41.7% compared with the untreated control. Previously treated NHDF cells with different concentrations of *T. minutus* extract (0.002%, 0.004%, 0.01%, and 0.02%, v/v) extensively diminished ROS levels by 23.4 ± 4.3%, 33.4 ± 3.9%, 43.7 ± 4.0%, and 57.2 ± 3.9%, respectively.



(**a**) (**b**)

Figure 5. ROS accumulation in (**a**) NHDF and (**b**) HaCaT cells after treatment with *T. minutus* extract for 24 h. Four stars (****) indicate statistically significant results ($p < 0.0001$).

Exposure of skin to ultraviolet light has been shown to have a number of deleterious effects, including photoaging, photoimmunosuppression, cancer, and photoinduced DNA damage [49–51]. While most UVC is absorbed by the ozone layer and does not manage to reach the Earth's surface, both UVA and UVB radiations have been implicated as carcinogens, although their modes of action are distinct [52,53]. Both types of UV light (A and B) are able to penetrate the skin to different depths, and hence affect different cells in the epidermis and dermis. UVB radiation is mainly absorbed by epidermal components such as proteins or DNA, whereas UVA radiation penetrates deeply into the skin and reaches the lower epidermis and dermal fibroblasts [54]. UVA radiation's toxicity mainly comes from oxidative damage skin cell components [55].

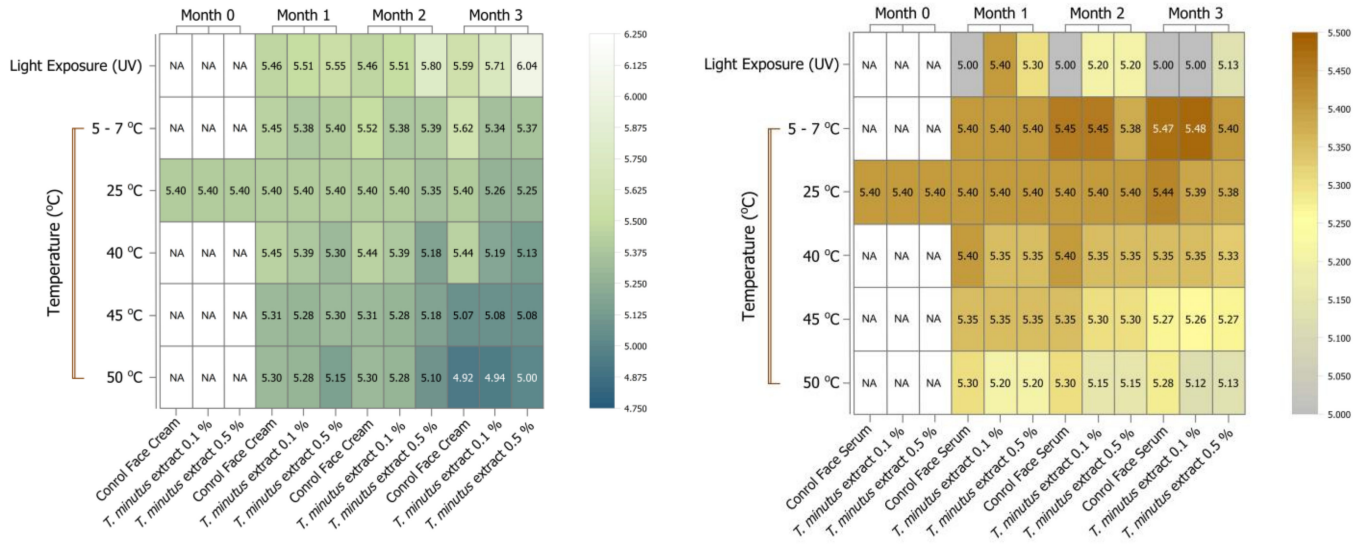
3.6. Evaluation of Cosmetics Formulations Containing T. minutus Extract and Stability Analysis

An oil in water (O/W) emulsion cream and an aqueous gel (face serum) containing 0.1% and 0.5% (*w*/*w*) *T. minutus* extracts were prepared. The composition of the formulations are listed in Tables 1 and 2. A cream and an aqueous gel without *T. minutus* extract were used as control. Emulsion systems of the O/W type attract significant attention for the development of a range of skincare products. The O/W emulsion is popular due to its washable and non-greasy properties. Furthermore, after application of the O/W emulsion to the skin, the water phase evaporates, causing the increase in the concentration of the bioactive compounds in the adhering film. As a consequence, the absorption of bioactive compounds into the skin is enhanced [35,36,56–58].

Quality control assessment tests were used for assessing the organoleptic characteristics and homogeneity of both formulations for three months. These properties were measured after storage at 5 °C, 25 °C, 40 °C, 45 °C, and 50 °C, and at daylight exposure (UV) at 0, 30, 60, and 90 days. The organoleptic characteristics showed no significant variations in their visual appearance during the study. Both formulations maintained not only their initial appearance but also their colour and scent. A homogeneity test was also conducted, in which all samples were evaluated for their visual appearance and touch affinity. Both formulations (cream or gel) maintain their visual appearance and touch affinity even after three months.

The pH stability of the O/W emulsion cream and the aqueous gel cosmetic formulations was also investigated. The measurement of pH is an important indicator of stability, especially in the case of creams. Figure 6a,b illustrates the pH measurements for three months under different storage conditions. The average pH of human skin ranges from 4.7 to 6.0, hence the pH of topical preparations must be in accordance with skin pH [59]. The results showed that the pH of the O/W emulsion cream and the aqueous gel did not exhibit significant variations over three months, suggesting that the formulation conditions were properly conducted. The stability of the pH values in both formulations within the acceptable range (4.7–6.0) indicates their stability and that no chemical changes occurred during storage.

The accelerated stability centrifugation test was also used for quality control assessment, since it provides a quick evaluation of the product's stability. Following centrifugation, no phase separation was observed when stored under different conditions (5–50 °C, daylight exposure), indicating that the O/W emulsion cream and the aqueous gel can be stable for a long time.



(**a**) (**b**)

Figure 6. Stability assessment of a cream (**a**) and of an aqueous gel (face serum) (**b**). pH measurements of different formulations were conducted at 0, 30, 60, and 90 days under different storage conditions (5–50 °C, daylight exposure). NA; Not Applicable.

4. Conclusions

In the present work, we provided the proof-of-concept basis for the application of *T. minutus* extracts for the preparation of cosmetic products. Bioactivity assessment revealed that *T. minutus* extracts exhibit high TAC along with significant elastase inhibitory activity. They do not display any significant cytotoxicity effect to NHDF and HaCaT, while they display significant protection from UVA-induced cell damage by decreasing oxidative stress. In addition, *T. minutus* extracts exhibit significant anti-inflammatory effects through the inhibition of the LPS-induced TNFα, IL-6, and IL-8 expression on human NHDF and HaCaT cells. Our findings underline that *T. minutus* extract is a promising bioactive ingredient with potential use as a raw material in cosmetics. Microalgae are a popular and valuable source of bioactive molecules in the health and wellness industry. The launching of new or underused microalgae strains with high bioactivity is desired to support the development of innovative new cosmetic products for the health and wellness industry. The expansion of new products from natural 'green' sources, such as microalgae, have significant potential in the area of bio-based products and procession.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cosmetics10030075/s1, Table S1: The primer sequences used for RT-qPCR, (TNFα, IL-6, IL-8 and ACT).

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Abbreviations

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, Ferric Reducing Antioxidant Power; HaCaT, cultured human keratinocyte cells; IL-6, interleukin-6; IL-8, interleukin-8; LPS, Lipopolysaccharide; NHDF, Normal Human Dermal Fibroblasts; TNFα, Tumor Necrosis Factor alpha; TAC, Total Antioxidant Capacity; ROS, Reactive Oxygen Species

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