

Article

Heat Stress Mitigation by *Haematococcus lacustris* Extract: Evidence from HaCaT Keratinocytes and *Caenorhabditis elegans*

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Abstract

Rising temperatures and the occurrence of heat waves due to climate change can increase the risk of various skin disorders. Moreover, elevated temperatures worsen oxidative damage and inflammation caused by other climate change stressors, such as UV exposure. Consequently, there is growing interest in innovative solutions to protect skin health from the effects of pollution and climate change stressors. Among natural cosmeceuticals, carotenoids are recognized for their antioxidant and anti-inflammatory properties. This study evaluated the thermoprotective effects of *Haematococcus lacustris* (the microalga formerly called *Haematococcus pluvialis*) extract (HLE), which is considered the richest natural source of carotenoid astaxanthin, against acute hyperthermia, which mimics the conditions of heat waves. In addition, we separately assessed the effects of HLE against UVA and hydrogen peroxide stress, complementing the antioxidant profile of the extract under study. The evaluation was conducted using in vitro tests on human HaCaT keratinocytes and the nematode *Caenorhabditis elegans*, which is a model organism sensitive to environmental stressors. The treatment of HaCaT keratinocytes with HLE counteracted the intracellular formation of reactive oxygen species and cytotoxicity induced by hyperthermia, UVA, and hydrogen peroxide exposure. Under the same experimental conditions, HLE also restored the impaired expression of stress-sensitive genes, such as matrix metalloproteinase-1, in HaCaT keratinocytes and promoted wound closure mimicking the process of re-epithelization. Lastly, experiments in *C. elegans* confirm that HLE reduces heat stress-induced oxidative damage and preserves motility, supporting a systemic protective effect consistent with dietary uptake of the extract. These findings suggest that HLE, rich in carotenoid astaxanthin, can protect keratinocytes against oxidative damage and cytotoxicity induced by thermal stress, indicating its potential role in mitigating thermal aging.

Keywords: astaxanthin; *Haematococcus lacustris*; heat stress; oxidative stress; UVA exposure; skin health; thermoprotection



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1. Introduction

Climate change has widespread effects on human health, particularly on the skin, the organ most exposed to the external environment. This exposure makes the skin highly

vulnerable to climate-related stressors, including rising temperatures and heat waves, which can disrupt its homeostasis and lead to various skin diseases [1–3]. Heat waves exacerbate the risk of inflammatory skin conditions, including atopic dermatitis, acne vulgaris, and psoriasis [1,2]. Additionally, higher temperatures can impair the skin's ability to repair and regenerate, further exacerbating oxidative damage and inflammatory responses triggered by other stressors, such as UV exposure [4,5].

Higher temperatures can also accelerate the aging process of the skin, a phenomenon often referred to as “thermal aging”. This phenomenon increases the production of enzymes such as matrix metalloproteinases-1 (MMP-1), which degrade collagen and elastic fibers, leading to the formation of wrinkles [6].

There is increasing interest in innovative solutions to mitigate the effects of climate change, such as rising temperatures and heat waves, on skin health [5]. Natural cosmeceuticals, which contain plant-derived compounds, can help reduce oxidative stress and inflammation and may treat various skin conditions [7]. Among phytochemicals, carotenoid astaxanthin (AST) has shown potential thermoprotective effects [8]. However, there are currently no studies evaluating these effects mediated by AST specifically at the skin level.

Haematococcus lacustris (HL), formerly called *Haematococcus pluvialis*, is a unicellular green microalga and a natural source of AST. This microalga is primarily composed of AST monoesters and diesters, but it also contains other valuable carotenoids, including β -carotene, canthaxanthin, and lutein [9,10]. AST is known for its health benefits and dermatology applications, including photoprotection and anti-inflammatory effects [9,11]. This microalga is sensitive to environmental stress, and studies have shown that higher temperatures increase AST accumulation, thereby helping it survive heat stress [12–14]. In terms of human health, in vivo studies in rats have shown that AST increases systemic thermotolerance by activating the NRF2-ARE antioxidant pathway and modulating anti-inflammatory signaling via the PI3K/AKT, ERK, and NF- κ B pathways [12]. Moreover, AST reduces reactive oxygen species (ROS) production in murine myoblasts exposed to heat stress, further supporting its role in improving cellular thermotolerance [8].

In this study, we evaluated the thermoprotective effects of a *Haematococcus lacustris* extract (HLE). This extract is mainly characterized by monoesters of AST combined with various fatty acids, including linoleic, linolenic, oleic and stearidonic acids. It also contains minor components such as AST diesters, lutein, canthaxanthin, and β -carotene, as reported in our previous characterizations [15–17]. We specifically assessed the ability of HLE to counteract oxidative damage induced by acute hyperthermia, which closely mimics the conditions of heat waves. Furthermore, we evaluated the activity of HLE against other environmental stressors, such as UVA and hydrogen peroxide (H_2O_2), independently, to complete the evaluation of the antioxidant profile of HLE.

Our experiments used human HaCaT keratinocytes and the model organism *Caenorhabditis elegans*. HaCaT keratinocytes represent the epidermis, which is the essential outer layer of the skin exposed to environmental stressors. The use of *C. elegans* allowed us to evaluate the thermal protective effects of HLE in a more complex biological system, as its skin consists of a simple epidermal epithelium covered by a cuticle [18].

2. Materials and Methods

2.1. Algal Cultivation

The cultivation of HL (Girod-Chantrans) Rostafinski was carried out as previously described by Rizzardi et al. [15]. The algal strain HP5 (isolated in Ravenna, Italy, in 2014) was grown in 1.5 L flasks using CHU13 medium, at a temperature of 21 ± 1 °C, light intensity of 100 μ mol of photons per $m^2 s^{-1}$ and a 16 h light: 8 h dark cycle. After 25–30 days of cultivation, vegetative cells were exposed to stress conditions (450–500 μ mol of photons per $m^2 s^{-1}$, 25 ± 2 °C, and

nutrient starvation at 3-fold dilution) to induce cyst formation. AST-accumulating red cysts were obtained, and after 30–40 days, they were used to prepare the HLE.

2.2. Preparation and Characterization of HLE

HLE was performed as reported by Rizzardi et al. [15]. The red cysts were collected by centrifugation at $2550 \times g$ for 10 min at 4 °C. After removing the supernatant, the algal pellet was freeze-dried. The pellet was then extracted with a mixture of cyclohexane/acetone/ethanol (CAE 2/1/1) for 48 h at room temperature. After filtration, the solvent was evaporated under vacuum. The carotenoid-enriched extract obtained was suspended in CAE (5 mL). An aliquot (0.1 mL) was withdrawn, diluted in CAE (1 mL), and analyzed spectrophotometrically (UV/VIS/NIR, JASCO V-650, JASCO, Tokyo, Japan) at 530 nm. A calibration curve prepared with standard astaxanthin in the free form ($0.5\text{--}25 \mu\text{g mL}^{-1}$) was used for quantification, and HPLC-MS analyses were used for qualitative identification of the astaxanthin monoesters in the extracts, as previously reported [15]. The compositional characterization of the HLE batch used in this study is reported in Figure S1. The total extract was divided into sub-aliquots, dried, and stored in the dark to avoid degradation.

2.3. Cell Culture

Human keratinocyte cells, HaCaT, were purchased from Cell Lines Service (CLS, Eppeheim, Germany) and routinely grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U mL⁻¹ penicillin, and 50 $\mu\text{g mL}^{-1}$ streptomycin at 37 °C in a humidified incubator with 5% CO₂ as previously described. For the experiments, stock solutions of HLE and synthetic AST (purity > 95%, Merck KGaA, Darmstadt, Germany) were prepared in dimethyl sulfoxide (DMSO) at 20 mg mL⁻¹. The stock solutions were further diluted in complete medium to obtain the desired concentrations of HLE, with a maximum of 1% DMSO, which did not affect HaCaT cell viability. Control conditions, however, received an equivalent percentage of DMSO without HLE or AST, in order to account for any potential solvent-related effects.

2.4. Stress Exposure

For heat stress experiments, cell plates were immersed up to the medium level in a thermostatic water bath (Julabo SW22, Julabo GmbH, Seelbach, Germany) maintained at 42 or 44 °C for 30 or 60 min, with moving water. Control cell plates were kept in a separate thermostatic water bath at 37 °C for the same time intervals. The cultures were covered with Parafilm to prevent evaporation during the treatment. For nematode experiments, agar plates were placed in a preheated incubator (Memmert Incubator I, Memmert GmbH + Co. KG, Schwabach, Germany) maintained at either 37 °C or 35 °C for 4 h. Control nematodes were kept in a parallel incubator at 20 °C for 4 h.

UVA irradiation was achieved with a bank of two Philips TLK 40W/10R fluorescence tubes (Sara s.r.l., Varese, Italy), which emit energy in the range of 350–400 nm and peak at 365 nm. These tubes were positioned to deliver 4.095 mW cm^{-2} of UVA, as measured by a radiometer (Oriel Instruments, Stratford, CT, USA) and reported in a previous study [19,20]. These fluorescent tubes operate cold, meaning they do not emit heat radiation and do not significantly alter the temperature of the surrounding environment. Cells and nematodes were exposed to the irradiation without the plate lid at room temperature. The UVA doses administered in different experiments were 5, 20, and 40 J, corresponding to exposure times of 20, 80, and 160 min, respectively.

2.5. Determination of Intracellular ROS Formation

HaCaT cells were seeded in 96-well plates at 3×10^4 cells/well and incubated at 37 °C in 5% CO₂. After 24 h of incubation, cells were incubated with the 2'-7' dichlorodihy-

drofluorescein diacetate (H₂DCF-DA) probe for 30 min. After removing the probe, HLE 6.25–12.5 µg mL^{−1} in Hanks' Balanced Salt Solution (HBSS) was added to each well, and simultaneously, cells were exposed to one of the following stress conditions: heat stress at 44 or 46 °C for 30 min, exposure to H₂O₂ at 100 µM, or UVA stress at 5 J cm^{−2}. The set of control cells was maintained at 37 °C for the heat stress experiment and kept in the dark for the UVA stress experiment. At the end of the exposure, ROS production was quantified by fluorescence reading (excitation at 485 nm and emission at 535 nm) using the VICTOR™ X3 multilabel plate reader (PerkinElmer). The fluorescence of HLE at the concentrations used was also measured without cells to eliminate any potential fluorescence contribution to the evaluation of ROS production.

2.6. Determination of Total Antioxidant Activity (TAA) in Cytosol and Membrane

The TAA was measured on both the cytosolic and membrane-enriched fractions as previously reported [19]. Briefly, HaCaT cells were seeded in culture dishes at 4×10^6 cells/dish for 24 h at 37 °C in 5% CO₂. At the end of incubation, cells were treated with HLE (6.25 µg mL^{−1}) for 2 h. After washing with cold phosphate-buffered saline (PBS), cells were detached using a cell lifter, collected in 1 mL of PBS and centrifuged for 10 min at 10,000 rpm at 4 °C. The supernatant was removed, and after washing with PBS, the pellet was finally reconstituted in 600 µL of 0.05% Triton X-100. Cells were then homogenized and allowed to stand at 4 °C for 30 min. The solution was centrifuged at 14,000 rpm for 15 min at 4 °C to extract the cytoplasmic fraction. The remaining pellet was then solubilized in 400 µL of 1% Triton X-100 and centrifuged again under the same conditions to obtain the membrane fraction. Small amounts were removed to determine the protein concentration using the Bradford method. TAA in cell fractions was determined by the decoloration of the radical cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), in terms of quenching of absorbance at 740 nm. Data for each sample were compared with the concentration–response curve of a standard antioxidant, such as Trolox (a water-soluble vitamin E analogue) and expressed as µmol of Trolox Equivalent Antioxidant Activity per mg of protein (µmol TE mg^{−1} protein).

2.7. Determination of Cytotoxicity

For the evaluation of cytotoxicity induced by heat stress, HaCaT cells were seeded in 96-well plates at 2×10^4 cells/well and incubated for 24 h at 37 °C in 5% CO₂. Subsequently, cells were treated with HLE (6.25–12.5 µg mL^{−1} in DMEM 2% FBS) and simultaneously exposed to heat stress at 44 °C for 30 min. After removing the treatment, fresh DMEM supplemented with 2% FBS was added to each well for 24 h of recovery at 37 °C. Cell viability was evaluated by the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to its insoluble formazan. The amount of formazan was measured (570 nm, reference filter 690 nm) using the multilabel plate reader VICTOR™ X3 (PerkinElmer, Waltham, MA, USA). The cytotoxicity was calculated by the formula:

$$\text{cytotoxicity (\%)} = 100 - \frac{\text{OD treated cells}}{\text{OD control cells}} \times 100$$

OD: optical density (absorbance) measured at 570 nm.

The absorbance of HLE at the concentrations used was also measured without cells to eliminate any potential absorbance contribution to the cytotoxicity calculation.

For the evaluation of cytotoxicity induced by UVA stress, HaCaT cells were seeded in 60 mm dishes at 1.5×10^6 cells/dish and incubated for 24 h at 37 °C in 5% CO₂. Then, cells were treated with HLE at 3.125 µg mL^{−1}, prepared in HBSS, and simultaneously exposed

to UVA stress (40 J/cm²). After irradiation, cells were allowed to recover for 24 h in DMEM 10% FBS and counted using eosin staining. The cytotoxicity was calculated by the formula:

$$\text{cytotoxicity (\%)} = 100 - \frac{\text{number of alive cells}}{\text{number of total cells}} \times 100$$

2.8. Determination of Cell Migration

Cell migration was assessed using a scratch assay, as previously described [21,22]. HaCaT cells were seeded in 24-well plates at 3×10^5 cells mL⁻¹. Once confluence was reached, cells were uniformly scraped with a 200-μL pipette tip across the well. Following wounding, each well was washed with PBS and treated with HLE (3.125 μg mL⁻¹) in DMEM with 2% FBS. One plate was exposed at 44 °C, while the control plate was maintained at 37 °C. After 1 h, the treatment was removed and replaced with 1 mL of DMEM 2% FBS. The scratched region was photographed immediately and 18 h after scratching using a Nikon microscope (Nikon Instruments S.p.a., Florence, Italy) at 20× magnification. Three wells were analyzed for each experimental condition. Total scratch area was analyzed using NIS-Elements imaging software version 6 (Nikon Instruments S.p.a.). Wound width (%) was calculated by comparing the scratch area at 0 h and 18 h using the formula:

$$\text{Wound width (\%)} = \frac{\text{Area 18 h}}{\text{Area 0 h}} \times 100$$

2.9. Determination of Gene Expression

Gene expression levels of MMP-1 were evaluated using quantitative real-time polymerase chain reaction (qRT-PCR). For heat stress, HaCaT cells in a 75 cm² flask at confluence were treated for 30 min with HLE (12.5 μg mL⁻¹ in DMEM 2% FBS) and stressed in a water bath at 44 °C. After stress, the treatment was removed, and new, fresh DMEM 2% FBS medium was added for 24 h of recovery. For UVA stress, HaCaT cells were seeded in 60 mm dishes at 1×10^6 cells/dish and incubated for 24 h at 37 °C in 5% CO₂. Then, cells were treated with HLE (12.5 μg mL⁻¹ in DPBS) and exposed to UVA (40 J cm⁻²). A recovery was then performed in DMEM 10% FBS. Afterwards, the cell suspension was pelleted, and RNA was extracted using the PureLink RNA Mini Kit (Life Technologies, Carlsbad, CA, USA), and 1 μg of RNA was used to synthesize cDNA using the Super-Script VILO MasterMix (Invitrogen, Carlsbad, CA, USA). PCR reactions were performed in triplicate in a 20 μL volume in a 96-well plate. The PCR mixture was prepared by combining 2 μL of cDNA (10 ng μL⁻¹), 10 μL of SYBR Select Master Mix (Invitrogen), and primers at a final concentration of 300 nM. The amplification conditions included 3 min at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C, with a melting curve analysis from 65 to 95 °C for five acquisitions/°C. β-Actin and glyceraldehyde-3-phosphate dehydrogenase protein (GAPDH, Life Technologies) were used as reference genes, and results were calculated using the 2⁻(ΔΔC_q) method. The primers used were: sense, 5'-TCTGACGTTGATCCAGAGAGCAG-3' and antisense, 5'-CAGGGTGACAC-CAGTGACTGCAC-3' for Matrix metalloproteinase-1 (MMP-1); sense, 5'-GCGAGAAGATGACCCAGATC-3' and antisense, 5'-GGATAGCACAGCCTGGATAG-3' for β-Actin; sense, 5'-GGTCGGAGTCAAC-GGATTTG-3' and antisense, 5'-GGAAGATGGTGATGGGATTTC-3' for GAPDH.

2.10. C. elegans Strain and Maintenance of Worms

The strain N2 standard wild-type was obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota, MN, USA). The strain was grown in NGM (0.3% NaCl, 2% agar, 0.25% peptone, 5 mg mL⁻¹ cholesterol, 1 M K₂HPO₄, 1 M MgSO₄) agar plates and fed with the bacterium Escherichia coli OP50, distributed in a thin layer over

the agar. HLE treatments were performed on OP50-seeded NGM plates, and no evident alterations of the bacterial lawn were observed under the experimental conditions. All worm strains were maintained at 20 °C, according to the standard protocol as described elsewhere [23].

For the experiments, a stock HLE solution was prepared in DMSO at 20 mg mL⁻¹. The stock solutions were further diluted in M9 1X Buffer to obtain the desired concentrations of HLE in a maximum of 2% DMSO, which did not affect *C. elegans* viability. Control conditions, however, received an equivalent percentage of DMSO without HLE, in order to account for any potential solvent-related effects.

2.11. Determination of ROS Formation and Touch-Provoked Responsiveness in *C. elegans*

To evaluate the effect of HLE on oxidative stress induced by hyperthermia and UVA in worms, the formation of ROS was assessed in N2 wild-type worms. L1 stage worms were treated on NGM/OP50 plates with 200 or 400 µg mL⁻¹ HLE for 48 h. HLE solutions were spread evenly onto the agar surface, and the solvent was allowed to evaporate before worm transfer. After treatment, worms reached the late L4/young adult stage, transferred to fresh NGM/OP50 plates, and exposed to heat stress at 37 °C for 4 h, while control worms were maintained at 20 °C. Meanwhile, another group of L1 stage worms was treated on NGM/OP50 plates with 200 or 400 µg mL⁻¹ HLE for 48 h. The worms were then transferred to fresh NGM/OP50 plates and exposed to UVA irradiation (20 or 40 J cm⁻²) equipped with a bank of two Philips TLK 40W/10R fluorescence tubes emitting 350–400 nm energy, while control worms were kept in darkness. After 24 h of recovery following hyperthermia and UVA exposure, worms from each plate were collected, washed and centrifuged twice using 1X M9 buffer. Then, the worms were incubated with 500 µL of 100 µM H₂DCF-DA probe for 1 h in the dark at 20 °C. After two washes with M9, approximately 5 µL of the worm solution was placed on 2% agarose pads, immobilized with a drop of 10 mM sodium azide, and then analyzed by fluorescence microscopy using a Nikon Eclipse Ti-U inverted fluorescence microscope at 20× magnification. Fluorescence intensity was quantified for the region of interest (ROI). A total of 10 untreated/treated 1-day-old adult animals were analyzed per condition, and each experiment was performed three times, for a total number of at least 30 nematodes.

Touch-provoked movement was also evaluated in the worms exposed to UVA before incubation with the fluorescent probe by applying a gentle mechanical stimulus with a platinum wire. Worms displaying movement in response to the stimulus were scored as touch-responsive.

2.12. Thrashing Assay in *C. elegans*

To evaluate the effect of HLE on worm motility, thrashing behavior was assessed in N2 wild-type worms. L1 stage worms were treated on NGM/OP50 plates containing HLE (100–200 µg mL⁻¹) for 48 h and then transferred to fresh NGM/OP50 plates and exposed at 35 °C for 4 h. After exposure, plates were transferred at 20 °C for a 24 h recovery period. Before scoring, each animal was immersed in 1 mL of M9 1X buffer for 30 s to acclimatize to the liquid medium. Thrashes were counted for 1 min under a stereomicroscope, and the average number per worm was calculated. For each experimental condition, ten worms per plate were used in triplicate.

2.13. Statistical Analysis

Statistical analyses were performed using one-way ANOVA, followed by Bonferroni's post hoc test for HaCaT cells and Tukey's post hoc test for *C. elegans*, with $p < 0.05$ considered statistically significant. Student's *t*-test was used when comparing only two experimental groups. All analyses were performed using PRISM 9 software (GraphPad Software, La

Jolla, CA, USA). Data for HaCaT cells are reported as the mean $\pm$ standard deviation (SD) of at least three independent biological replicates, while data for *C. elegans* are reported as the mean $\pm$ standard error of the mean (SEM) of at least three independent biological replicates (~ 10 animals/condition per replicate; total $n \geq 30$ /condition).

3. Results

3.1. Physical–Chemical Characteristics of HLE

The yield of HLE (48 ± 2 wt%) and AST content in the HLE (8.0 ± 0.4 wt%) were in line with results reported in previous studies using the same strain [16,17]. AST monoesters were identified by LC-MS, considering the molecular weight. Monoesters of linoleic (C18:2) and linolenic acid (C18:3) were the most abundant peaks, followed by oleic (C18:1) and stearidonic acid (C18:4) monoesters (Figure S1). The monoester with palmitic acid (C16:0) was the only C16-ester detected, while minor signals were ascribable to AST diesters, as previously found [16,17]. The ratio between AST monoesters and diesters was 5.2 ± 0.4 , in line with the literature [16,24].

3.2. HLE Reduces Oxidative Damage Induced by Hyperthermia in Keratinocytes

Several studies on hyperthermia in human cells indicate that the temperature level near $43\text{--}44$ °C significantly impacts cellular antioxidant defenses, protein stability, and DNA integrity, leading to cell death [25,26]. In particular, this temperature induces heat stress at the cellular level, affecting the distribution of plasma membrane proteins. This alteration alters plasma membrane permeability, resulting in a calcium spike and disrupting the mitochondrial membrane potential, which changes the redox status of the cells and shifts it toward oxidative stress [27].

Since different cell types can show varying sensitivities to hyperthermia, we initially established hyperthermia conditions that induced oxidative damage in HaCaT cells. This was done by measuring intracellular ROS levels and assessing the cytotoxicity in these cells.

The formation of ROS in HaCaT cells was measured after 30 min of exposure to high temperature using the $\text{H}_2\text{DCF-DA}$ assay. Meanwhile, the cytotoxicity in HaCaT cells was evaluated 24 h after recovery at 37 °C, following the same 30 min hyperthermic exposure, using the MTT assay. We compared two temperatures, 42 °C and 44 °C, above the normal maintenance temperature of 37 °C, in HaCaT cell cultures. As shown in Figure 1A,B, increasing the temperature to 42 °C resulted in the formation of ROS but did not cause cytotoxicity in the HaCaT cells. In contrast, increasing the temperature to 44 °C led to a significant rise in both the ROS formation and cytotoxic effects. This suggests that the higher levels of ROS exceeded the ability of HaCaT cells to counteract them, resulting in cytotoxicity and confirming the temperature threshold reported in the literature [25].

Therefore, we used 44 °C in subsequent experiments to evaluate the ability of HLE to counteract oxidative damage induced by heat stress in HaCaT cells. At a normal cultivation temperature of 37 °C, treatment of HaCaT cells with HLE showed that concentrations of $25 \mu\text{g mL}^{-1}$ and above decreased cell viability (Figure 2A). Therefore, we selected non-cytotoxic concentrations of 6.25 and $12.5 \mu\text{g mL}^{-1}$ for subsequent thermoprotection experiments. Intracellular ROS formation in HaCaT cells induced by heat stress was determined after simultaneous exposure to HLE at 6.25 and $12.5 \mu\text{g mL}^{-1}$ and 44 °C for 30 min. The results show that HLE significantly reduced ROS formation caused by the high temperature at all tested concentrations (Figure 2B). This antioxidant activity was further confirmed against the intracellular ROS formation triggered by H_2O_2 in the presence of HLE $12.5 \mu\text{g mL}^{-1}$ in HaCaT cells (Figure 2C). Considering that AST is the predominant carotenoid present in HLE, we also evaluated its activity by comparing the antioxidant effects of HLE ($12.5 \mu\text{g mL}^{-1}$) with those of synthetic AST ($1 \mu\text{g mL}^{-1}$),

corresponding to the AST content present in HLE (8 wt%). Both treatments were evaluated under hyperthermic conditions (44 °C) and after H₂O₂-induced oxidative stress. HLE and synthetic AST showed comparable efficacy in reducing intracellular ROS formation under both experimental conditions (Figure S2).

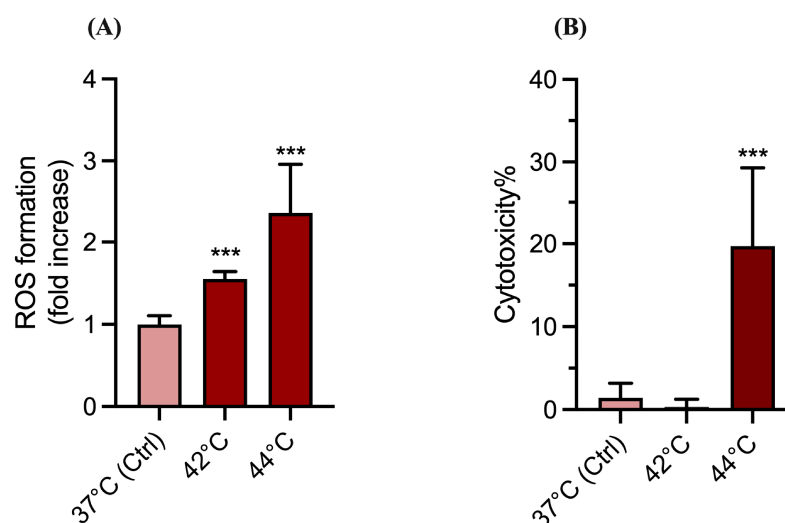


Figure 1. Effects of oxidative damage induced by hyperthermia in HaCaT cells. (A) The cells were exposed to 37, 42 and 44 °C for 30 min. After these exposures, the intracellular ROS levels were evaluated using the H₂DCF-DA assay. (B) The cells were exposed to temperatures of 37, 42, and 44 °C for 30 min. After a 24 h recovery period at 37 °C, the cytotoxicity was evaluated using the MTT assay. Data are expressed as mean $\pm$ SD (n. 4). *** $p < 0.001$ vs. control cells at 37 °C; one-way ANOVA followed by Bonferroni's post hoc test.

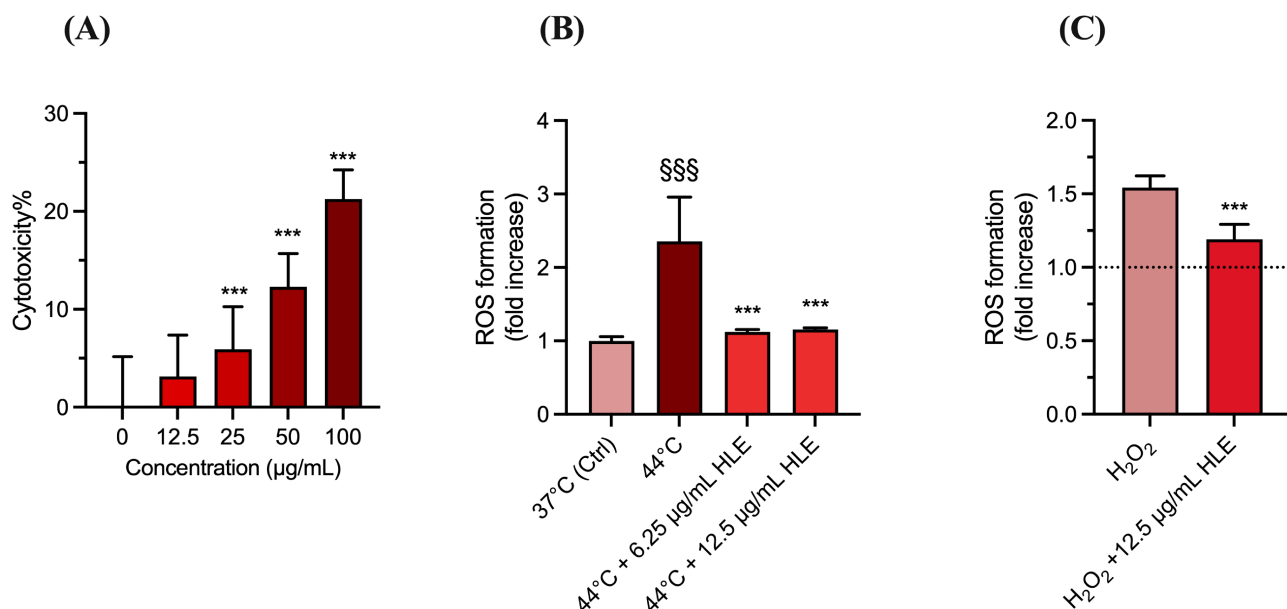


Figure 2. Effects of HLE on cytotoxicity and intracellular ROS formation induced by hyperthermia in HaCaT cells. (A) Cells were treated with HLE (12.5–100 µg mL^{−1}). After a 24 h recovery period at 37 °C, the cytotoxicity was evaluated using the MTT assay. (B) Cells were simultaneously exposed to HLE (6.25–12.5 µg mL^{−1}) and 44 °C for 30 min. (C) Cells were simultaneously exposed to HLE (12.5 µg mL^{−1}) and H₂O₂ (100 µM) for 30 min. After these exposures, ROS formation was evaluated using the H₂DCF-DA assay. Data are expressed as mean $\pm$ SD (n. 4). SSS $p < 0.001$ vs. control cells at 37 °C; *** $p < 0.001$ vs. cells exposed to either 44 °C or H₂O₂; one-way ANOVA followed by Bonferroni's post hoc test; Student's *t*-test.

The ability of HLE to counteract intracellular ROS formation prompts an evaluation of its contribution to total antioxidant activity at both membrane and cytosol levels, measured as equivalents of $\mu\text{mol TE}$ for mg of proteins. This approach allows assessment of HLE enrichment at these two distinct subcellular levels and provides insight into the potential bioavailability of antioxidant phytochemicals at these levels. Membrane and cytosol fractions from HaCaT cells treated with HLE ($12.5 \mu\text{g mL}^{-1}$) for 2 h showed a significant increase in TAA compared to untreated HaCaT cells. This finding suggests the movement of carotenoids from HLE into both the membrane and the cytosol of keratinocytes (Figure 3).

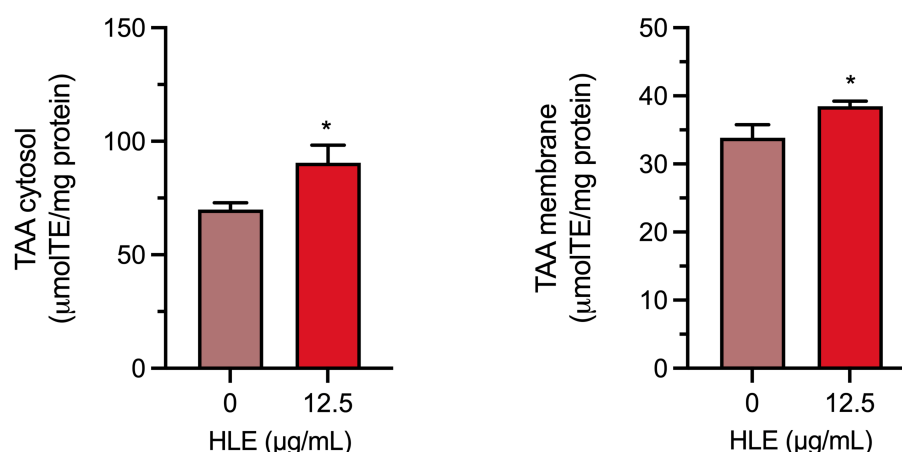


Figure 3. Effects of HLE on TAA of membrane and cytosol in HaCaT cells. Cells were treated for 2 h with HLE ($12.5 \mu\text{g mL}^{-1}$), and then ABTS radical scavenging activity was evaluated in the cytoplasmic and membrane fractions using the ABTS assay. Data are expressed as micromoles of Trolox per milligram of protein. Data are expressed as mean $\pm$ SD (n. 4). * $p < 0.05$ vs. untreated cells, Student's *t*-test.

Remarkably, 6.25 and $12.5 \mu\text{g mL}^{-1}$ HLE also mitigated the cytotoxicity in HaCaT cells induced by 44°C exposure, highlighting its cytoprotective potential against thermal cellular damage through its antioxidant activity (Figure 4A). Subsequently, we adopted the same hyperthermia exposure conditions and $12.5 \mu\text{g mL}^{-1}$ HLE, with a 24 h recovery at 37°C , to evaluate the gene expression of matrix metalloproteinase-1 (MMP-1) using real-time PCR. MMP-1 in keratinocytes is a biomarker of cellular stress that acts as a driving force in skin aging [6].

As shown in Figure 4B, HLE treatment significantly counteracted the heat stress-induced increase in MMP-1 gene expression, reducing it to baseline levels when heat stress was absent. Excessive collagen matrix degradation by MMP-1 impairs keratinocyte function, hindering their ability to spread and migrate, which delays wound healing and disrupts essential tissue repair signaling [28].

Given the ability of HLE to reduce MMP-1 levels under heat stress, we also evaluated its impact on wound closure of HaCaT cells using a scratch assay. This assay also mimics the re-epithelialization and wound healing processes in acute and chronic wounds [21,29]. HaCaT cells treated with HLE ($\mu\text{g mL}^{-1}$) exhibited significantly greater wound closure than untreated cells under 44°C stress conditions after 18 h (Figure 4C), a result that parallels the reduced cytotoxicity observed under the same conditions (Figure 4A), suggesting that improved cell viability contributes to the enhanced wound closure phenotype.

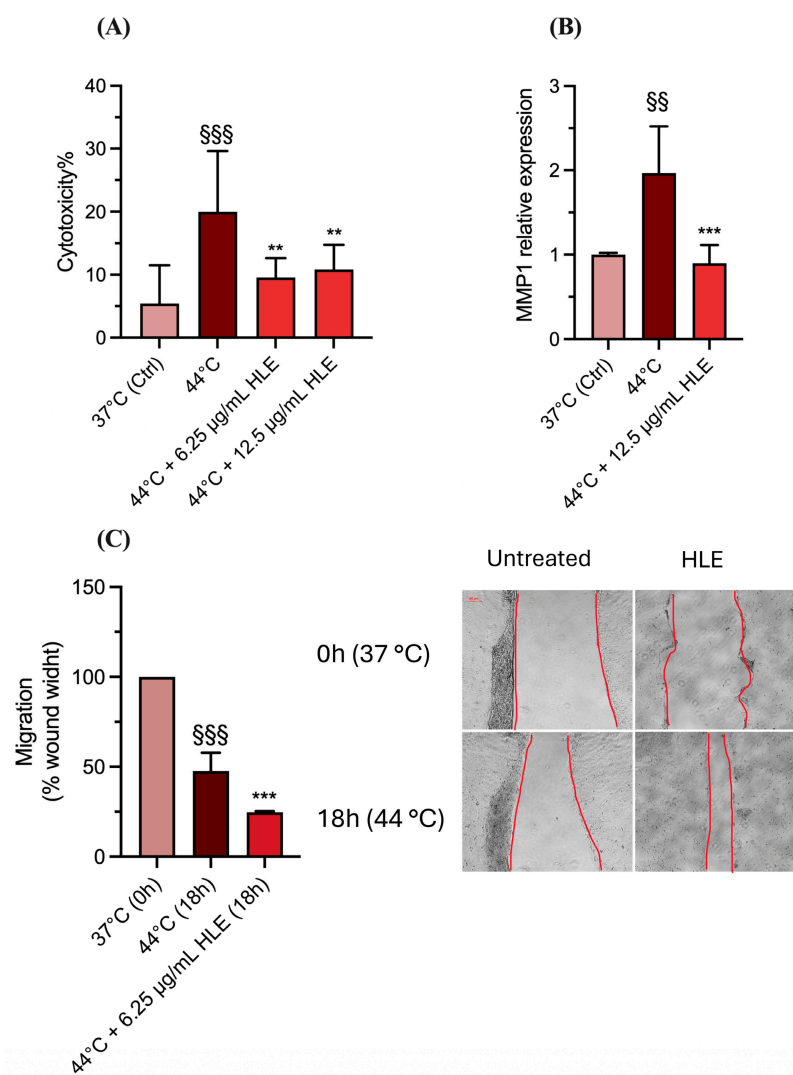


Figure 4. Effects of HLE on cytotoxicity, MMP-1 activation, and impaired wound closure induced by hyperthermia in HaCaT cells. **(A)** Cells were simultaneously exposed to HLE (6.25–12.5 µg mL^{−1}) and 44 °C for 30 min. After a 24 h recovery period at 37 °C, cell viability was evaluated using the MTT assay. **(B)** Cells simultaneously exposed to HLE (12.5 µg mL^{−1}) and 44 °C for 30 min. After a 24 h recovery period at 37 °C, the gene expression of MMP-1 was determined using real-time PCR. **(C)** Cells treated with HLE (6.25 µg mL^{−1}) and exposed to 44 °C for 1 h, and then maintained in standard conditions. Scratch assay was performed, and wound width was measured at t0 and after 18 h. Images were acquired using a 20× objective. In all experiments, a set of control cells was maintained at 37 °C for cell cultivation. Data are expressed as mean ± SD (n. 4). §§ $p < 0.01$ and §§§ $p < 0.001$ vs. control cells at 37 °C; ** $p < 0.01$ and *** $p < 0.001$ vs. cells exposed to 44 °C; one-way ANOVA followed by Bonferroni's post hoc test.

3.3. HLE Reduces Oxidative Damage Induced by UVA in Keratinocytes

UVA (320–400 nm) significantly contributes to UV-related skin damage, and high temperatures can amplify its oxidant, cytotoxic, and genotoxic effects [30]. Therefore, we assessed the ability of HLE to protect HaCaT cells from oxidative damage caused by UVA exposure. We evaluated the same parameters of oxidative damage associated with hyperthermia, such as intracellular ROS formation and cytotoxicity, following UVA exposure in the presence of various concentrations of HLE (6.25 and 12.5 µg mL^{−1}). The concentrations of 6.25 and 12.5 µg mL^{−1} HLE significantly reduced ROS formation in HaCaT cells after exposure to 5 J cm^{−2} of UVA (Figure 5A). Additionally, a concentration of 12.5 µg mL^{−1} of HLE mitigated the decrease in cell viability after a 24 h recovery period

following exposure to 40 J cm^{-2} of UVA (Figure 5B). In addition to assessing cell viability, we evaluated the gene expression of MMP-1 in HaCaT cells that were simultaneously exposed to $12.5 \mu\text{g mL}^{-1}$ of HLE and 40 J cm^{-2} of UVA. Remarkably, HLE significantly attenuated the UVA-induced increase in MMP-1 gene expression (Figure 5C).

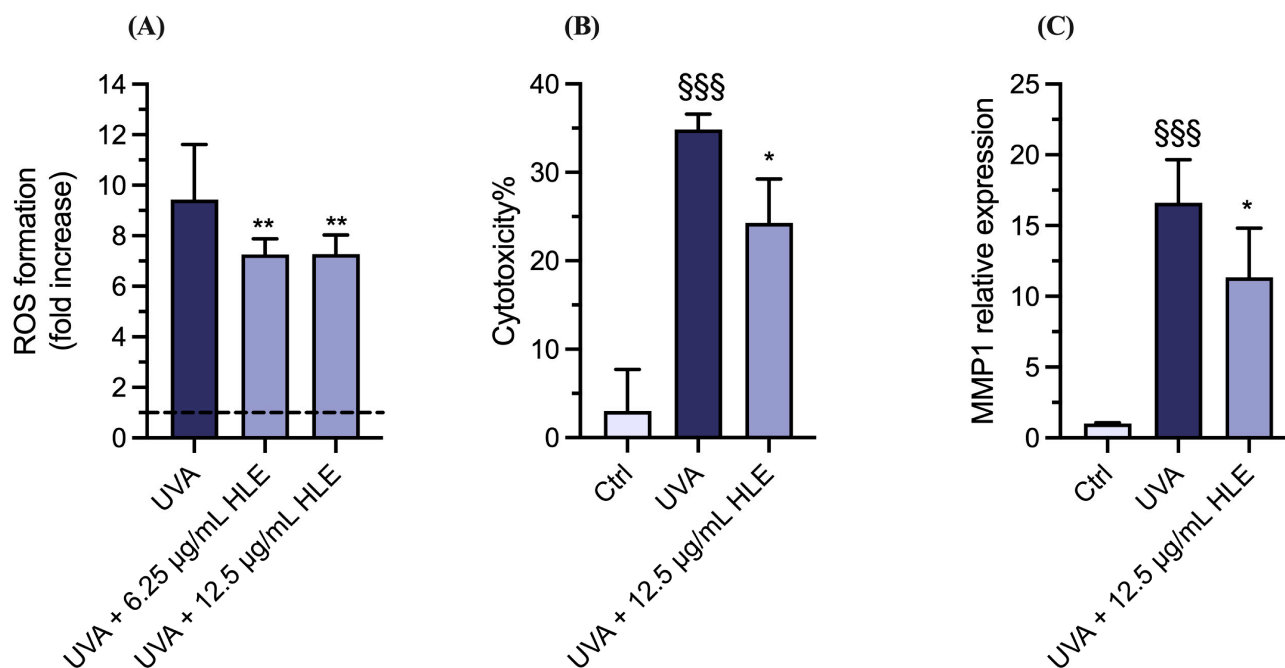


Figure 5. Effects of HLE on the reduction of UVA-induced oxidative damage, cytotoxicity, and MMP-1 expression in HaCaT cells. (A) Cells simultaneously exposed to HLE ($6.25\text{--}12.5 \mu\text{g mL}^{-1}$) and UVA (5 J cm^{-2}). After this exposure, ROS formation was evaluated using the $\text{H}_2\text{DCF-DA}$ assay. (B) Cells simultaneously exposed to HLE ($12.5 \mu\text{g mL}^{-1}$) and UVA (40 J cm^{-2}). After a 24 h recovery period, the cell viability was evaluated by eosin staining. (C) Cells simultaneously exposed to HLE ($12.5 \mu\text{g mL}^{-1}$) and UVA (40 J cm^{-2}). After a 24 h recovery period, the gene expression of MMP-1 was determined using real-time PCR. In all experiments, a set of control cells was not exposed to UVA. Data are presented as mean $\pm$ SD (n. 4). §§§ $p < 0.001$ vs. cells not exposed to UVA; * $p < 0.05$, ** $p < 0.01$ vs. cells exposed to UVA; one-way ANOVA followed by Bonferroni's post hoc test.

These findings suggest that HLE can counteract ROS formation and the resulting oxidative damage induced by UVA exposure. The evaluation of the UV–Vis absorption spectrum demonstrated no significant absorption in the UVA region (320–400 nm), indicating that HLE is unlikely to provide direct shielding from UVA radiation under the experimental conditions used (Figure S3).

3.4. HLE Increases Heat Stress Resistance in *C. elegans*

C. elegans has a cuticle made up of a cross-linked protein matrix along with external lipid layers. This structure shares functional similarities with the human stratum corneum, which is the outermost layer of the skin. As a result, *C. elegans* is a valid model for studying processes related to skin barrier function, although in the present study, exposure occurred via the diet rather than through the cuticle [31,32]. To evaluate resistance to thermal stress, we treated L1-stage wild-type N2 nematodes with $200 \mu\text{g mL}^{-1}$ HLE for 48 h at 20°C . After this treatment, the nematodes were subjected to heat stress at 37°C for 2 h, while controls were maintained at 20°C . After a 24 h recovery period, we assessed ROS production using the $\text{H}_2\text{DCF-DA}$ probe. Heat stress significantly increased ROS levels in nematodes compared with those kept at 20°C ($p < 0.001$). The treatment with HLE reduced this increase in ROS, confirming its strong antioxidant properties (Figure 6A). Notably, these

findings indicate that HLE reduces heat-induced oxidative stress in nematodes, which may be consistent with a supportive role in cuticle-mediated protection; however, no direct measurements of cuticle permeability or structural integrity were performed.

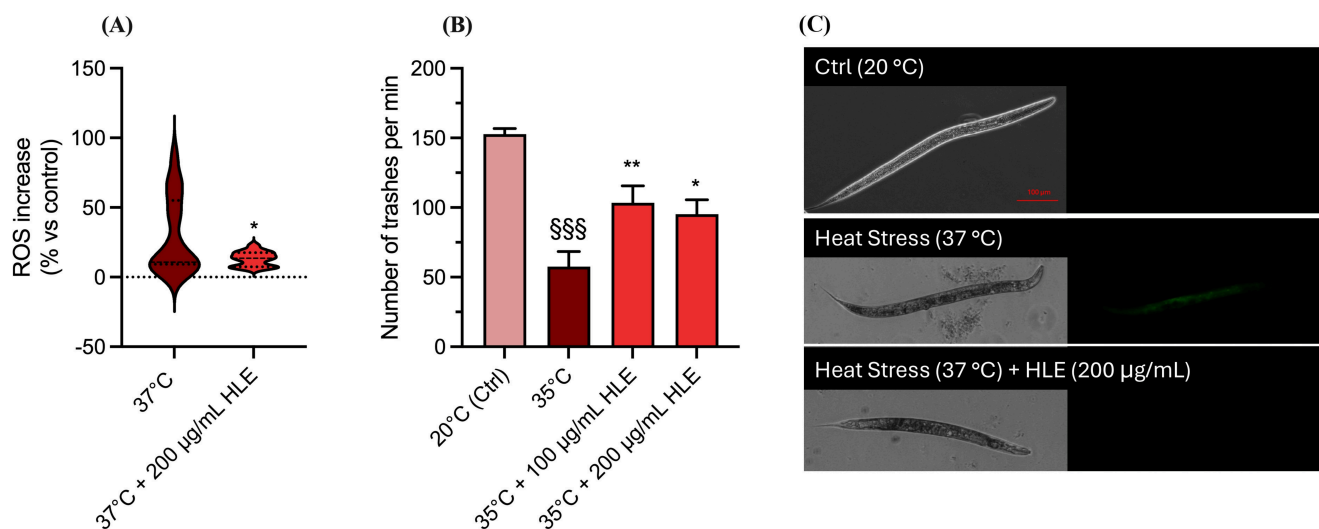


Figure 6. HLE reduces ROS formation and restores the motility impaired by hyperthermia in *C. elegans*. (A) N2 wild-type nematodes in the L1 stage were exposed to HLE 200 µg mL⁻¹ for 48 h at 20 °C, then exposed to a temperature of 37 °C for 2 h. After 24 h of recovery, ROS production was assessed using the H₂DCF-DA assay. ROS levels are expressed as a percentage increase compared to the control at 20 °C. (B) N2 wild-type nematodes were exposed to HLE (100–200 µg mL⁻¹) from the L1 for 48 h at 20 °C, then exposed to a temperature of 35 °C for 4 h. After 24 h of recovery, a thrashing test was performed. Results are expressed as mean ± SEM (n. 30 nematodes). \$\$\$ $p < 0.001$ vs. standard condition; * $p < 0.05$, ** $p < 0.01$ vs. heat-stressed nematodes; one-way ANOVA followed by Tukey's post hoc test. (C) Representative images of oxidative stress shown via fluorescence microscopy in wild-type *C. elegans* subjected to heat stress (2 h at 37 °C), with or without HLE treatment at 200 µg mL⁻¹ (20× magnification).

Furthermore, to evaluate the functional effect of HLE on nematode motility, we conducted a thrashing test, which involves counting the number of complete lateral movements during swimming. Wild-type N2 nematodes at the L1 stage were treated with HLE (100 and 200 µg mL⁻¹) for 48 h at 20 °C, followed by a 4 h heat stress at 35 °C. After 24 h of recovery at 20 °C, the results show a significant improvement in motility in the treated nematodes compared to stressed controls, which show a marked reduction in movement (Figure 6B).

Meanwhile, ROS formation was evaluated in N2 wild-type nematodes treated with HLE 200 or 400 µg mL⁻¹ for 48 h and then exposed to 20 J cm⁻² of UVA. HLE demonstrated the ability to significantly reduce the ROS levels, with a maximum inhibition of 55% at 400 µg mL⁻¹ (Figure 7A). Notably, the 400 µg mL⁻¹ of HLE also counteracted the reduction in touch-provoked responsiveness induced by exposure to 40 J cm⁻² of UVA (Figure 7B), confirming the shielding effect of astaxanthin against abiotic stresses.

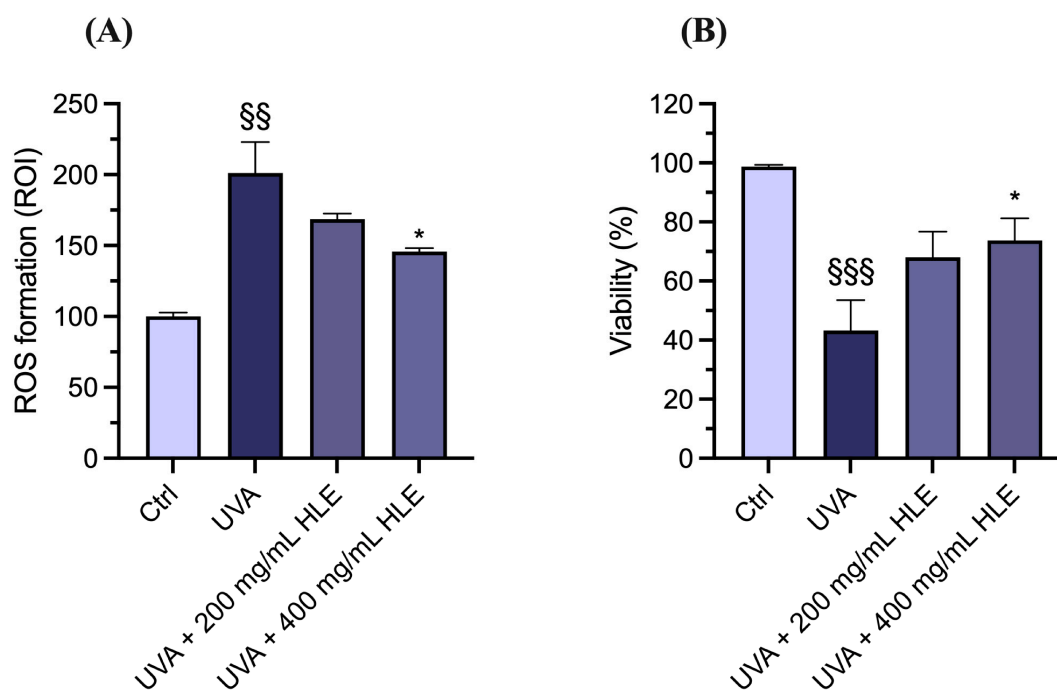


Figure 7. Effects of HLE on ROS formation and touch-provoked responsiveness in *C. elegans* exposed to UVA irradiation. **(A)** N2 wild-type nematodes in the L1 stage were exposed to HLE 200 or 400 $\mu\text{g mL}^{-1}$ for 48 h at 20 °C, then exposed to 20 J cm^{-2} UVA. After 24 h of recovery at 20 °C, ROS formation was assessed using the $\text{H}_2\text{DCF-DA}$ assay. ROS levels, indicated by fluorescence intensity, were quantified in the region of interest (ROI) in worms. **(B)** N2 wild-type nematodes in the L1 stage were exposed to HLE 200 or 400 $\mu\text{g mL}^{-1}$ for 48 h at 20 °C, then exposed to 40 J cm^{-2} UVA. After 24 h of recovery, the touching assay was performed. Results are expressed as mean $\pm$ SEM (n. 30 nematodes). §§ $p < 0.01$ and §§§ $p < 0.001$ vs. standard condition; * $p < 0.05$ vs. nematodes exposed to UVA; one-way ANOVA, followed by Tukey's post hoc test.

4. Discussion

Environmental stressors, including extreme temperatures, UV radiation, and pollution, significantly affect skin health and contribute to skin disease [33]. Elevated temperatures can impair skin barrier function, alter lipid organization, and accelerate aging by degrading collagen and elastin. Mechanisms such as mitochondrial dysfunction and epigenetic changes can contribute to these effects [5]. These pathways are linked by ROS formation and the depletion of cellular antioxidants, such as glutathione and vitamin E, which exacerbates oxidative stress [5,34]. Phytochemicals offer potential as nutraceuticals and cosmeceuticals to help maintain skin homeostasis disrupted by these environmental stressors [35].

In this context, our findings demonstrate that HLE protects against skin damage induced by environmental stressors, such as hyperthermia. In particular, the HLE decreased intracellular ROS formation and cytotoxicity induced by heat stress in HaCaT keratinocytes.

Hyperthermia can affect the phospholipid bilayer of the cellular membrane, increasing its fluidity and permeability. This can lead to lipid peroxidation, ROS formation, depletion of antioxidant defenses, and cell death [36,37]. In this regard, we evaluated the effects of HLE on TAA in HaCaT keratinocytes. Following a short treatment, TAA showed a slight increase at the membrane level but a significant increase at the cytosolic level, suggesting that the antioxidant carotenoids in HLE could be distributed differently between these two levels, with a predominant presence in the cytosol. Among the carotenoids found in HLE, astaxanthin monoesters are the most abundant forms. Their amphipathic structures are reported to enable orientation across the membrane, which has been associated with more effective protection against lipid peroxidation compared with beta-carotene and lutein,

which are oriented parallel to the membrane surface [38]. Based on this structural feature, it has been proposed that such orientation may reinforce the membrane and enhance its capacity to maintain cellular homeostasis under thermal stress [6,39]. Notably, the significant increase in antioxidant activity observed at the cytosolic level is consistent with, but does not directly demonstrate, a transfer of astaxanthin monoesters across the membrane and their enrichment in the cytosol of keratinocytes. A recent study comparing the intracellular antioxidant activity of algal-based astaxanthin and free-form astaxanthin, using cholesterol esterase in HaCaT keratinocytes, found no significant difference between them. This result supports the antioxidant contribution of astaxanthin esters found in algae to the HaCaT keratinocytes [40]. However, direct evidence of membrane translocation and subcellular carotenoid localization in our experimental model is lacking, and further validation of this hypothesis is needed.

The thermoprotective effects of HLE against the intracellular ROS formation induced by elevated temperature may also help to prevent oxidative damage that contributes to extrinsic skin aging. This damage can lead to the breakdown of collagen and elastin in fibroblasts, as well as impair the integrity of the skin barrier. These processes often result in coarse wrinkles, a loss of elasticity, and sagging, all of which compromise skin integrity [33]. Notably, HLE has demonstrated the ability to reduce the gene expression of MMP1 induced by heat stress in HaCaT keratinocytes. This transcriptional effect is consistent with previous studies reporting reduced MMP-1 protein levels and collagen preservation in human dermal fibroblasts [40]. In addition, HLE has shown the ability to counteract the cytotoxicity on HaCaT keratinocytes and promote wound closure under higher temperature conditions. These results further emphasize the protective role of HLE against oxidative and cytotoxic damage induced by high temperature, and support its potential contribution as an antioxidant carotenoid in counteracting thermal aging-related processes [41,42].

Among environmental factors, UV exposure plays a significant role in oxidative damage, in a similar manner to higher temperatures, contributing to photoaging [5,39]. UV exposure increases the production of ROS, activating the MAPK signaling pathways. This activation leads to the upregulation of MMPs, enzymes that degrade collagen and other components of the extracellular matrix, thereby contributing to skin photoaging [40]. In this context, HLE demonstrated protective effects against UVA damage in HaCaT keratinocytes, similar to those observed under elevated temperatures. In particular, HLE reduces ROS formation, lowers MMP-1 levels, and mitigates the cytotoxicity associated with UVA exposure. Previous studies on dermal fibroblasts have shown that AST can scavenge ROS generated by radiation, interrupt lipid peroxidation and inhibit the phosphorylation of key cell signaling proteins, such as ERK and CREB [43]. These mechanisms have been proposed to subsequently reduce the expression of MMP-1 induced by UVA. Although the phosphorylation status of ERK/CREB was not assessed in the present study, the reduction in MMP-1 levels observed with HLE is consistent with a possible involvement of these pathways, suggesting that the carotenoid AST may contribute to the photoprotective effects observed with HLE [43]. A more recent study involving human subjects has shown that AST from *H. pluvialis* exhibits potent anti-ultraviolet (UV) effects due to its antioxidative properties on the skin and its appendages [44].

Our findings in HaCaT keratinocytes were also confirmed in *C. elegans* under conditions of hyperthermia and UVA exposure. In *C. elegans*, the external cuticle is essential for movement, nutrition, and protection from environmental stressors [31]. It consists of a collagenous extracellular matrix covered with a lipid-rich layer [45], serving as a permeability barrier akin to human skin. This allows for low absorption of compounds via passive diffusion [31,46]. The compounds are primarily absorbed through the gastrointestinal tract

after oral intake, with systemic concentrations comparable to those in mice, aiding in dose translation from nematodes to mammals [47].

In this regard, treating *C. elegans* with HLE provided protection against oxidative stress by reducing the ROS formation induced by hyperthermia. In addition, it helped maintain locomotor activity, a crucial function in *C. elegans* that is often affected by oxidative damage. This suggests that HLE exerts a systemic thermoprotective effect in *C. elegans*, consistent with its dietary route of administration, although these effects also need to be demonstrated in animal models.

These findings align with the existing literature, which shows that astaxanthin can extend the lifespan of *C. elegans* and enhance its resistance to heat stress, an effect reported to be mediated by activation of the DAF-16 pathway, an ortholog of the human FOXO (Forkhead box O) transcription factor pathway involved in longevity, stress resistance, metabolism, and tumor suppression in humans [48]. Although DAF-16/FOXO activation was not directly measured in the present study, the observed improvements in redox status and locomotor activity under heat stress are consistent with the possible involvement of this pathway. Therefore, these findings suggest that HLE may mitigate the effects of environmental hyperthermia primarily through systemic antioxidant activity rather than a direct barrier-reinforcing mechanism.

5. Conclusions

This study highlights the thermoprotective effect of HLE against hyperthermia on skin damage. In particular, HLE counteracted the formation of intracellular ROS and cytotoxicity, reduced MMP-1 gene expression, and promoted wound closure in HaCaT keratinocytes under acute heat stress, suggesting its thermoprotective properties. Notably, these thermoprotective effects may be attributed to the antioxidant carotenoids present in HLE, which can distribute within the membrane, particularly in the cytosol of HaCaT keratinocytes, thereby contributing to the attenuation of oxidative stress under high-temperature conditions such as those associated with heat waves. This was further supported by findings in *C. elegans*, in which HLE improved redox status and motor function under heat stress. In addition, HLE showed protective effects against oxidative damage mediated by UVA irradiation in *C. elegans*, suggesting that its antioxidant activity is effective against various types of environmental stress when considered individually. Future studies that combine heat stress and UVA exposure will be essential to determine whether HLE can also protect against their potential interactive or cumulative effects on skin cells. Notably, the carotenoids found in HLE, especially the most abundant monoester astaxanthin, warrant further investigations for their role in thermoprotection. Clinical studies suggest that carotenoids accumulate in the skin, supporting their antioxidant potential in vivo [49]. This makes HLE a promising cosmeceutical for maintaining skin health and preventing thermal aging. In this regard, these findings support further investigation in three-dimensional reconstructed epidermis models to validate these effects and confirm their translational relevance to human skin.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/cosmetics13040214/s1>.

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Abbreviations

The following abbreviations are used in this manuscript:

DMSO	Dimethyl sulfoxide
H ₂ DCF-DA	2'-7' dichlorodihydrofluorescein diacetate
HL	<i>Haematococcus Lacustris</i>
HLE	<i>Haematococcus Lacustris</i> extract
AST	Astaxanthin
MMP-1	Matrix metalloproteinases-1
ROS	Reactive oxygen species

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