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Effects of lutein, phytoene and carotenoid-rich microalgal extracts on the epidermis of *Caenorhabditis elegans*

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ABSTRACT

Carotenoids are widespread bioactive compounds that can accumulate in the skin. Microalgae, such as *Chlorella sorokiniana* and *Dunaliella bardawil*, are a sustainable source of natural carotenoids. This study evaluates the effect of phytoene- and lutein-rich green microalgal extracts along with pure phytoene and lutein, on the epidermis of the nematode *Caenorhabditis elegans*. Wild-type and sensitised mutant backgrounds were used to examine the effect of the selected carotenoids on epidermal stem cells, which differentiate to give rise to mature epidermal, neuronal, and support cell types. We also assessed their impact on cuticle integrity, the protective outer layer secreted by epidermal cells. Results revealed that phytoene-enriched microalgae at 3 μg/mL significantly increased stem cell number and improved cuticle integrity (4.4- to 12.4-fold less permeable). Our findings support a role for carotenoids in the epidermis of *C. elegans*, with potential implications for future developments in dermocosmetics.

1. Introduction

Carotenoids are versatile natural pigments widely distributed in nature. Beyond their well-known antioxidant role, carotenoids are also involved in a range of biological processes, including vitamin A biosynthesis, antioxidant and anti-inflammatory responses (Meléndez-Martínez, 2019). Due to their versatility as colorants, antioxidants, bioactive compounds, and precursors of vitamin A, carotenoids are very important as components of different products intended for human consumption, such as functional foods, supplements, (nutri)cosmetics and nutraceuticals (Meléndez-Martínez et al., 2021).

Just a few carotenoids are colorless. A classic example is phytoene, which has been less studied than other common dietary carotenoids. However, this carotenoid has gained increasing attention in recent years due to its potential health-promoting properties, including antioxidant, photoprotection, and anti-inflammatory activities, as well as possible roles in reducing the risk of diseases such as skin condition, cardiovascular disorders, and certain types of cancer (Mapelli-Brahm & Meléndez-Martínez, 2021). Phytoene is found in many fruits and vegetables, such as tomatoes, carrots, apricots, citrus, or watermelon, among others (Mapelli-Brahm & Meléndez-Martínez, 2021).

Among systems to produce bioactive compounds, microalgae cultivation requires less land and water compared to traditional crops, which reduces pressure on natural resources. Furthermore, certain microalgae species have potential for biofuel production of bioactive compounds and environmental remediation, aligning with the ethos of sustainable and eco-friendly practices. Thus, microalgae are considered a sustainable source of bioactive compounds, such as carotenoids, which offer several benefits for human health (Mapelli-Brahm et al., 2023). Apart from sustainable sourcing, the adoption of sustainable solvents and extraction processes is also important for producing high-quality products with minimal environmental impact. The use of green techniques for carotenoid extraction, such as ultrasound-assisted extraction, enables higher yield recovery while reducing solvent consumption and extraction time compared to traditional extraction methods, such as maceration. The use of green solvents such as 2-methyltetrahydrofuran (MeTHF, also known as 2-methyloxolane) is also in demand; however, only a few studies have so far evaluated the use of this bio-based solvent for carotenoid extraction (Morón-Ortiz et al., 2024).

To assess the effect of natural products on health, animal models

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such as *Caenorhabditis elegans*, a microscopic nematode of approximately one millimeter in length, are particularly useful. *C. elegans* has emerged as an invaluable biological model in scientific research due to several notable advantages (Corsi et al., 2015). First, its short life cycle of about three to four days (with a lifespan ranging from 12 to 18 days) allows for rapid and efficient observation of effects over time. In addition, *C. elegans* shares several key biological pathways with mammals, including humans, which facilitates in some cases the extrapolation of the results obtained in this organism to more complex situations (Li et al., 2023).

The *C. elegans* extracellular cuticle is the outermost barrier of the organism, which is formed by a complex and multilayer structure approximately 0.5 μm thick. The main function of the cuticle is protecting the nematode from the external environment as well as facilitating maintenance of body morphology and locomotion (Sundaram & Pujol, 2024). The cuticle is composed of collagen, cuticlins, lipids, and glycoproteins. *C. elegans* cuticle is synthesized five times during its life cycle, i.e., at the end of embryogenesis before hatching, and before each molt during the four larval stages (Sundaram & Pujol, 2024). Seam cells, a group of epidermal stem cells located along the lateral sides of *C. elegans*, play a key role in cuticle production in two ways. First, they secrete the lateral ridges of the cuticle known as alae, and second, they generate more cells that join the secretory epidermis thereby helping it expand and produce the cuticle (Altun & Hall, 2002; Chisholm & Xu, 2012). L1 nematodes contain a total of 10 seam cells per lateral side, most of which divide symmetrically at L2 (to expand the stem cell pool) and asymmetrically (to generate differentiated daughter cells) once in every larval stage, thereby ending with 16 seam cells in the adult stage in a highly reproducible manner (Altun & Hall, 2002; Katsanos et al., 2017; Sulston & Horvitz, 1977). To visualise these cells, strains such as JR667 (*wIs51*) that contain a seam cell GFP marker in a wild-type (N2) background, allow phenotypic observation and characterisation under a fluorescence microscope (Ashley et al., 2021; Hintze et al., 2020). Previous genetic studies have identified numerous mutants with cell lineage anomalies that result in perturbed seam cell counts. These anomalies can influence the potential for cell proliferation or differentiation, the timing of specific cell divisions, or the process of division changing cell polarity or asymmetry (Ambros & Horvitz, 1984; Gorrepati et al., 2013; Hintze et al., 2021; Huang et al., 2009; Kagoshima et al., 2007; Katsanos et al., 2017; Koneru et al., 2021). For instance, *C. elegans rnt-1* mutants reduce seam cell number largely due to defects in the L2 symmetric division, which is converted to asymmetric (Kagoshima et al., 2007; Nimmo et al., 2005). This gene is homologous to the human RUNX transcription factor, part of the RUNX/CBFβ complex, which is involved in cell proliferation, differentiation, and survival (Coffman, 2003; Kagoshima et al., 2007; Nimmo & Woollard, 2008). Another example is the *egl-18* mutants, which show defective seam cell fate maintenance ultimately leading to a reduction in seam cell number. This gene encodes a GATA transcription factor, and is a target of the Wnt siganlling pathway playing a role in various aspects of animal development (Gorrepati et al., 2013; Koh & Rothman, 2001). Because Wnt signaling and seam cell biology are inextricably linked to cuticle development (Jackson et al., 2014), seam cell patterning mutants often show defective cuticle for example with interrupted alae. In addition, the *bus-19* mutant (T07F10.4) encodes a conserved membrane protein of the DedA family, whose precise biochemical role remains unclear. Mutations in *bus-19* cause severe cuticle abnormalities, including disrupted alae, strong lectin binding, bleach hypersensitivity, and increased surface permeability (Yook & Hodgkin, 2007). These phenotypes make *bus-19* a sensitive strain for evaluating epidermal barrier function.

Importantly, the *C. elegans* epidermis shows functional parallels with human skin. In both organisms, the outermost barrier is enriched in lipids and structural proteins that protect against environmental stress. Moreover, conserved signaling pathways, particularly Wnt, regulate epidermal proliferation and differentiation in nematodes as well as in mammals. These similarities reinforce the use of *C. elegans* as a translational model for studying compounds with potential cosmeceutical applications (Chisholm & Hsiao, 2012).Carotenoids are known to accumulate in skin and be involved in its health and appearance (Meléndez-Martínez et al., 2019), however, little is known about their influence on skin resistance, renewal, and permeability. In this sense, this study aimed to investigate the role of two eco-friendly extracts from phytoene-rich microalgae (*Chlorella sorokiniana* and *Dunaliella bardawil*) and two pure carotenoids (phytoene and lutein) on the epidermis of *C. elegans*.

2. Materials

2.1. Reagents

Tert-butyl methyl ether (HPLC-grade) and $CaCl_2$ were purchased from Honeywell (Seelze, Germany); MeTHF, KH_2PO_4, Na_2HPO_4, sodium azide, sodium hydroxide, and cholesterol from Sigma-Aldrich (Steinheim, Germany); methanol (HPLC-grade) and ethyl acetate (HPLC-grade) from VWR Chemicals (Leuven, Belgium); sodium chloride from Fisher Chemical (Hampton, EEUU). Agar, peptone, dimethylsulfoxide, Hoechst 33342, and LB media from Thermo Fisher Scientific (Hampshire, UK); $MgSO_4$ from Melford (Suffolk, UK); agarose from Appleton Woods (Birmingham, UK); phytoene standard and lutein standard from Extrasynthese (Genay, France).

2.2. Microalgae cultivation

Chlorella sorokiniana (211−32) was kindly provided by the Institute of Plant Biochemistry and Photosynthesis (IBVF-CSIC, Seville, Spain) from its algal collection. This alga was cultured photomixotrophically (100 μE m^2/s PAR) at 25 °C in modified liquid Tris-acetate phosphate (TAP) medium (León et al., 2005). *Dunaliella bardawil* (UTEX 2538) was obtained from the UTEX Culture Collection of Microalgae (University of Texas, Austin), and it was cultured in liquid modified Johnson's medium (Johnson et al., 1968) (2 M NaCl) under photoautotrophic conditions with CO_2-enriched aeration, continuous illumination, and agitation. For both microalgae, once optimal cell concentration was reached, the biomass was harvested by centrifugation, frozen at −80 °C, and subsequently lyophilized.

2.3. Enrichment of the microalga with phytoene

Cultures of *C. sorokiniana* in the middle of the exponential growth phase were harvested by centrifugation, resuspended in fresh TAP culture medium, divided into 2 L cultures, and incubated with 1 μg/mL of the herbicide norflurazon (NF) as described in previous studies (Morón-Ortiz, Mapelli-Brahm, León-Vaz, Benitez-González, et al., 2024).

D. bardawil was also subjected to an inhibitor of the carotenogenic pathway to force phytoene accumulation by following the procedure of León et al. (2005). Briefly, microalga cells were treated with the optimized amount of 10 μg/mL of the herbicide NF.

2.4. Extraction of carotenoids from microalgae

The algal carotenoid extracts were obtained by ultrasound-assisted extraction (frequency of 20 kHz, amplitude of 30 %, extraction time of 2 min, and a ratio of solvent-to-solid of 20 mL/g) using the green biosolvent MeTHF. Following ultrasonication, the samples were centrifuged, and the supernatant was transferred to another tube. The extraction process was repeated until the sample appeared colorless, Subsequently, the samples were concentrated using a rotary evaporator (Eppendorf Concentrator plus™, Eppendorf, Hamburg, Germany), either for use as supplement in *C. elegans* assays or for HPLC analysis.

We selected MeTHF as an eco-friendly solvent based on our previous studies on *C. sorokiniana* and *D. bardawil*, where it showed comparable or superior carotenoid recoveries compared to conventional solvents

(Morón-Ortiz, Mapelli-brahm, León-Vaz, Benítez-González, et al., 2024; Morón-Ortiz, Mapelli-Brahm, & Meléndez-Martínez, 2024). Furthermore, ultrasound-assisted extraction has been widely reported to improve microalgal cell wall disruption and carotenoid recovery compared to conventional extraction methods (Morón-Ortiz, Mapelli-Brahm and Meléndez-Martínez, 2024).

2.5. C. elegans culture and strains

C. elegans were maintained on nematode growth medium (NGM) seeded with *Escherichia coli* strain OP50, according to standard procedures (Stiernagle, 2006). The nematodes were synchronized by treatment of adult worms with bleach solution (50 μL NaOH (1 M), 20 μL of commercial bleach, and 30 μL distilled water), followed by washing the released eggs with M9 buffer. The JR667 strain, which contains a *scm::GFP* transgene (*wIs51*) or N2 was used as a reference strain. The mutant strains used in this study were MBA290 [*egl-18(ga97)*; *wIs51*], MBA1284 [*rnt-1(ok351)*; *wIs51*], and CB6338 [*bus-19*(e2912)].

2.6. Preparation of experimental plates

NGM plates of 55 mm were seeded with 200 μL of OP50 *E. coli* used as a food source and then, 48 h later, supplemented with 100 μL of DMSO (control) or 100 μL of phytoene-rich extracts from the microalgae, phytoene standard, and lutein standard at concentrations of 1 μg/mL and 3 μg/mL, diluted in DMSO. Worms fed only with OP50 were included as a baseline to confirm the proper performance of the assays in the absence of solvent, whereas DMSO-fed worms served as the vehicle control for all carotenoid treatments. Consequently, statistical analyses were performed against the DMSO group. The concentration of 1 μg/mL was chosen based on previous work conducted by the authors showing that this dose of carotenoids is effective and non-toxic in *C. elegans* (Morón-Ortiz, Karamalegkos, Mapelli-Brahm, Ezcurra and Meléndez-Martínez, 2024), while 3 μg/mL was included as a threefold higher dose to explore potential dose-dependent effects. These were left to dry for two days at room temperature. The supplemented plates were stored at 4 °C until needed. Supplemented plates were used within a maximum of two weeks. Eggs were placed directly on the supplemented plates, so that worms were exposed to the carotenoids or extracts continuously from hatching through development and adulthood until the assays were performed. The final concentration of DMSO on the plates was approximately 1 % (*v*/v). Although this concentration is at the upper end of the range typically used in *C. elegans* studies, it was necessary to ensure solubilization of all carotenoid treatments. A DMSO-only control was therefore included in every assay, and all comparisons were made against this group to account for any solvent-related effects.

2.7. Seam cell number assay

Standard seam cell scorings were carried out by placing approximately 40 synchronized nematodes on 3 % agar pads containing 100 μM sodium azide (NaN_3). A coverslip was then placed, and samples were examined using an AxioScope A1 (Zeiss) upright epifluorescence microscope. Seam cell counting was performed by focusing on one lateral side of each specimen at the end of the L4 stage, when divisions are completed. Seam cell number quantification was carried out in three independent experiments.

2.8. Bleaching sensitivity assay

Approximately 10 synchronized gravid day 2 adult nematodes were placed in 100 μL of commercial bleach solution (sodium hypochlorite, 4–4.9 % *w*/w NaClO, Teepol, UK). The time from when the worm was deposited in the bleach solution to the point of cuticle breakage (egg release) was recorded. Day-2 adults were used in this assay to ensure full cuticle maturation, which improves the robustness of the bleaching test. The experiment was performed three times in quadruplicates.

2.9. Cuticle permeability test

Approximately 50 synchronized day 1 adult nematodes were harvested and transferred to Eppendorf tubes. Day-1 adults were selected for this assay to minimize variability, since the cuticle is already functional but remains relatively uniform across individuals at this stage. To each tube, 1 mL of a Hoechst 33342 solution (1 μg/mL in M9 buffer) was added for staining. The samples were then gently shaken in the dark with a Stuart SB3 rotator (Cole-Parmer, UK) for 30 min at room temperature. After incubation, the worms were washed with M9 buffer. The nematodes were then placed on a microscope slide with 40 μL of 3 % agarose pad containing 100 μM sodium azide. A coverslip was mounted, and the samples were examined under a AxioScope A1 (Zeiss) upright epifluorescence microscope. Nematodes were classified, according to the penetration of Hoechst 33342 dye through the cuticle in the tail region, as blue-stained or non-blue-stained animals and this experiment was repeated three times.

2.10. HPLC analysis

Quantification of carotenoids was carried out using an Agilent 1260 Infinity II Prime U-HPLC instrument (Waldbronn, Germany) equipped with a diode array detector and a C_{30} column (3 μm, 150 × 4.6 mm) (YMC, Wilmington, NC). Carotenoid extracts were dissolved in 500 μL of ethyl acetate, and 10 μL were injected into the system for analysis. The wavelengths monitored were 285 nm for phytoene and 450 nm for the rest of the carotenoids. The mobile phase consisted of a mixture of methanol, *tert*-butyl methyl ether, and water, delivered at a flow rate of 1 mL/min via a linear gradient, as described in a study by Stinco et al. (2019). Carotenoid content was determined by external calibration as explained in that study. To determine the total carotenoid content, the individual carotenoid concentrations were summed.

2.11. Statistical analysis

Data analysis and statistical assessments were performed using R Studio (version 4.3.3). Normality was evaluated using the Shapiro-Wilk test, followed by the Kruskal-Wallis test for group comparisons. Post hoc analyses were conducted using Dunn's test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Carotenoid profile in two microalgal extracts

The main carotenoids found in *C. sorokiniana* were, in descending order, lutein, (15*Z*)-phytoene, β-carotene, α-carotene, (all-*E*)-phytoene, antheraxanthin, and (9*Z*)-β-carotene (Supplementary Fig. 1). In *D. bardawil* they were (15*Z*)-phytoene, lutein, zeaxanthin, β-carotene, (all-*E*)-phytoene, antheraxanthin, and (9*Z*)-β-carotene (Supplementary Fig. 1). The carotenoid profile of the extracts revealed that *C. sorokiniana* was particularly rich in lutein (119.44 μg/g) and that zeaxanthin was detected in *D. bardawil* (14.38 μg/g) but not in *C. sorokiniana*. Both species showed high levels of phytoene, mainly as (15*Z*)-phytoene, specifically 117.86 μg/g in *C. sorokiniana* and 99.68 μg/g in *D. bardawil*. The two phytoene isomers detected accounted for 47 % and 45 % of the total carotenoid content in *C. sorokiniana* and *D. bardawil*, respectively. The total carotenoid content was comparable between species (259.91 μg/g in *C. sorokiniana* and 246.34 μg/g in *D. bardawil*).

3.2. Treatment with carotenoids modulates seam cell number

First, it was assessed whether feeding nematodes with extracts of phytoene-enriched microalgae (at 1 and 3 μg/mL) or with pure phytoene

or lutein had any effect on the number of seam cells per lateral side in wild-type, and in *rnt-1(ok351)* and *egl-18(ga97)* animals used as sensitised mutant backgrounds. No statistically significant differences in seam cell counts per lateral side were observed between wild-type nematodes fed with pure phytoene at concentrations of 1 μg/mL or 3 μg/mL, or with pure lutein at 3 μg/mL, compared to the control group (DMSO) (Fig. 1A and Table 1). However, the number of seam cells per lateral side in nematodes fed with lutein at 1 μg/mL (16.02) was significantly higher than in control nematodes (15.86) ($p < 0.01$). The phytoene-enriched *D. bardawil* extract-fed nematodes showed a significant increase in seam cell numbers at both 1 and 3 μg/mL relative to the DMSO-fed nematodes (control) ($p < 0.05$) (Fig. 1A and Table 1). Nematodes fed with phytoene-rich *C. sorokiniana* extracts at 1 μg/mL did not show significant differences compared to the DMSO control; however, the average seam cell number of nematodes fed with *C. sorokiniana* extracts at 3 μg/mL was significantly higher than that found in control nematodes ($p < 0.05$) (Fig. 1A and Table 1).

In the *rnt-1(ok351)* mutant, higher variability in seam cell number among nematodes compared to JR667 was found (Fig. 1B and Table 1). Nematodes fed with the phytoene enriched microalgae extracts or phytoene at 1 μg/mL did not show significant differences in seam cell number (12.18 (*C. sorokiniana*), 12.34 (*D. bardawil*), and 12.59 (pure phytoene) seam cells per lateral side) compared to DMSO (12.50). However, dosing lutein at 1 μg/mL led to significant differences compared to the control, with an average seam cell number of 12.78 ($p < 0.05$) (Fig. 1B and Table 1). The treatments at a concentration of 3 μg/mL led in all cases to significant differences compared to the control. Among them, the phytoene-enriched extract from *C. sorokiniana* produced the highest seam cell average (13.65), followed by lutein (13.46). The phytoene standard resulted in a mean seam cell count of 13.34, while the phytoene-enriched *D. bardawil* extract showed a slightly lower mean of 13.10. Moreover, all the tested samples at 3 μg/mL demonstrated a significant increase in seam cell number compared to their respective concentrations at 1 μg/mL ($p < 0.01$ for lutein and $p < 0.001$ for the rest of the samples) (Fig. 1B and Table 1).

Seam cell number quantification in the *egl-18(ga97)* mutant background also showed a higher variability in seam cell number among nematodes compared to the wild type (Fig. 1C and Table 1). At 1 μg/mL, none of the tested samples differed significantly from the DMSO control. However, at 3 μg/mL, all samples exhibited a statistically significant increase in the number of seam cells per lateral side relative to DMSO (11.53 cells) ($p < 0.001$). Seam cell counts ranged from 12.53 in phytoene-treated nematodes to 12.90 in those treated with phytoene-enriched *C. sorokiniana* (12.90 seam cells per lateral side). Additionally, all treatments at 3 μg/mL showed significantly higher seam cell numbers than at 1 μg/mL ($p < 0.001$) (Fig. 1C and Table 1).

3.3. Treatment with carotenoids influences cuticle integrity

The cuticle integrity of *C. elegans* was assessed via two previously established assays (Loer et al., 2015; Sandhu et al., 2021). First, sensitivity to bleach exposure involves measuring the time to cuticle rupture upon sodium hypochlorite treatment. To test the effect of the different treatments (extracts and pure carotenoids at 1 and 3 μg/mL) on cuticle robustness, we used wild-type and *egl-18(ga97)* mutants, which are known to have cuticle defects (Koh & Rothman, 2001). Strikingly, phytoene-enriched *C. sorokiniana* extracts at 3 μg/mL significantly extended the cuticle rupture time to 12.71 min, which was 1.57 times longer relative to the control treated with DMSO (8.07 min) ($p < 0.001$) (Fig. 2A and Table 2). Similarly, pure phytoene and phytoene-enriched *D. bardawil* extracts, both at 3 μg/mL, also exhibited prolonged rupture times (11.49 min and 11.30 min, respectively), that is, increases of 1.42 and 1.40-fold compared to the DMSO control ($p < 0.001$). However, although pure lutein at 3 μg/mL extended the cuticle rupture time to 8.63 min compared to the control, this difference was not statistically significant (Fig. 2A and Table 2). On the other hand, at 1 μg/mL, all samples tested showed a statistically significant improvement in cuticle rupture time compared to DMSO ($p < 0.001$). Specifically, phytoene extended the rupture time to 10.66 min (1.32-fold increase), phytoene-enriched *C. sorokiniana* to 10.46 min (1.30-fold increase), lutein to 9.59 min (1.19-fold increase), and phytoene-enriched *D. bardawil* extract to 9.21 min (1.14-fold increase). Furthermore, at a concentration of 3 μg/mL, both phytoene-enriched microalgae significantly enhanced cuticle resistance to rupture compared to their respective extracts at 1 μg/mL ($p < 0.001$) (Fig. 2A and Table 2).

In a parallel experiment using the *egl-18(ga97)* mutant of *C. elegans*, significant enhancements in cuticle resistance to rupture were observed when the nematodes were fed with all the tested samples at both concentrations of 1 and 3 μg/mL ($p < 0.05$) (Fig. 2B and Table 2). The highest improvement was observed with the phytoene-enriched *D. bardawil* extract dosed 3 μg/mL, which extended the time to cuticle rupture to 8.51 min (1.35 times longer than that observed in the control) ($p < 0.001$). Additionally, the treatment of lutein at 3 μg/mL prolonged the rupture time to 8.40 min, which was 1.33 times longer than that of the control ($p < 0.001$). The treatment with the phytoene-enriched *C. sorokiniana* extract at 3 μg/mL led to a rupture time of 8.23 min, i.e. 1.31 times longer than that of the control ($p < 0.001$). Phytoene at 3 μg/mL also demonstrated enhanced cuticle resistance, extending the rupture time to 8.13 min, i.e., 1.29 times that of the control ($p < 0.001$) (Fig. 2B and Table 2).

At 1 μg/mL, the treatment with the phytoene-enriched *D. bardawil* extract resulted in an average cuticle rupture time of 7.67 min (1.22-fold longer compared to control, $p < 0.001$). This was followed by phytoene-enriched *C. sorokiniana* extract (7.47 min, 1.19-fold extension, $p < 0.001$), lutein (7.00 min, 1.11-fold extension, $p < 0.01$) and phytoene (7.09 min, 1.13-fold extension, $p < 0.05$). Moreover, in all samples, dosage at 3 μg/mL led to statistically significant enhancements in cuticle resistance compared to the treatments at 1 μg/mL; this effect was especially pronounced for phytoene and lutein ($p < 0.001$) (Fig. 2B and Table 2).

Second, the permeability of the cuticle can be assessed by evaluating the penetration of a cell-permeant, nuclear Hoechst stain through the cuticle. Here, we used *bus-19* mutants as a sensitised mutant background because *bus* mutants in *C. elegans* are known to show cuticle permeability defects (Yook & Hodgkin, 2007). Interestingly, significant differences compared to the DMSO control were observed when the nematodes were exposed to any of the treatments tested ($p < 0.001$ in all cases) (Fig. 3 and Table 3). Lutein at 3 μg/mL showed the highest resistance to Hoechst penetration, with only 3.33 % of the nematodes showing a blue tail. This was 12.41-fold lower relative to the control, where 41.33 % of the nematodes had a blue tail (Fig. 3 and Table 3). The treatment with phytoene-enriched *C. sorokiniana* microalgae extract at 3 μg/mL resulted in 4.00 % of blue-stained nematodes, representing a 10.33-fold reduction compared to the control. Phytoene-enriched *D. bardawil* extracts at 1 and 3 μg/mL resulted in 4.67 and 5.33 % blue-stained nematodes, respectively, corresponding to 8.85-fold and 7.75-fold decreases relative to DMSO, respectively. Similarly, phytoene at 1 μg/mL showed a 5.33 % of blue-stained nematodes, i.e. a 7.75 -fold reduction compared to the DMSO control. Both phytoene-enriched *C. sorokiniana* microalgae extract at 1 μg/mL and lutein at 3 μg/mL showed 6.67 % blue-stain nematodes (6.20-fold reduction compared to DMSO). Finally, dosing phytoene at 3 μg/mL resulted in 9.33 % of blue-stained nematodes (4.43-fold reduction compared to the control sample) (Fig. 3 and Table 3).

4. Discussion

We sought to explore the impact of two phytoene-rich microalgae extracts and two pure carotenoids, phytoene and lutein, on the epidermis and cuticle integrity in *C. elegans*. It is well-documented that some carotenoids (β-carotene, canthaxanthin, lutein, among others) and carotenoid-rich products have positive effects on skin health and, in

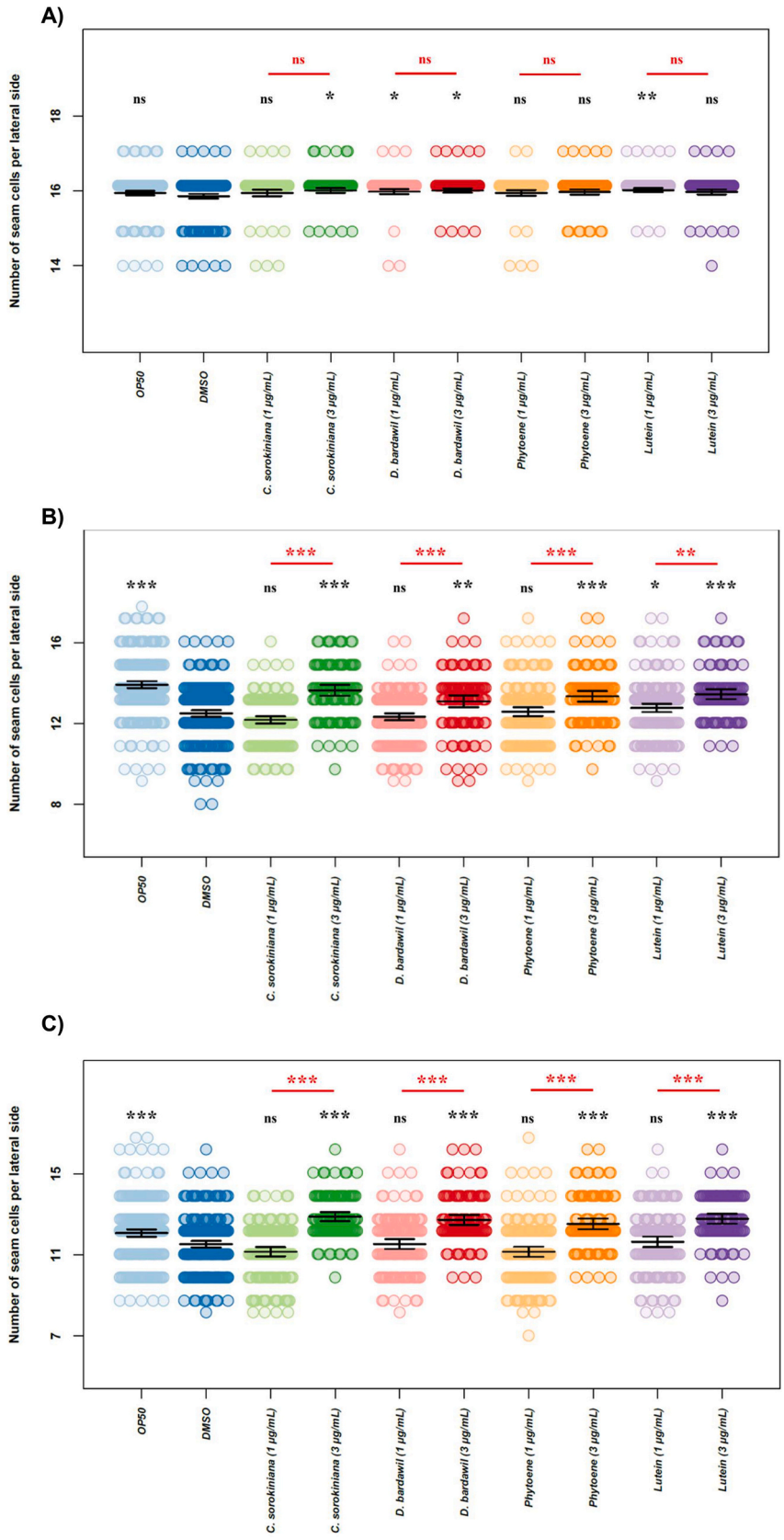

Fig. 1. Number of seam cells per lateral side in wild type (JR667 strain) **(A)**, *rnt-1(ok351)* mutants **(B)** and *egl-18(ga97)* mutants **(C)** treated with *C. sorokiniana* and *D. bardawil* extracts and pure phytoene and lutein. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. $n = 140$ worms per condition. Black stars show significant differences between each treatment and control (DMSO). The error bars in the figures represent the standard deviation (SD). Among the same compounds, red stars show significant differences between both concentrations evaluated (1 and 3 µg/mL). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$;

ns: not significant. OP50: *Escherichia coli* OP50; DMSO: dimethylsulfoxide. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

Table 1
Number of seam cells per lateral side in wild-type, *rnt-1(ok351)* and *egl-18(ga97)* mutants treated with *C. sorokiniana* and *D. bardawil* extracts and pure phytoene and lutein.

	JR667	MBA1284 - *rnt-1(ok351)*	MBA290 - *egl-18 (ga97)*
OP50	15,94 ± 0,41^{ns}	13,96 ± 1,48***	12,08 ± 1,49***
DMSO	15,86 ± 0,47	12,50 ± 1,36	11,53 ± 1,32
***C. sorokiniana* (1 μg/mL)**	15,94 ± 0,43^{ns}	12,18 ± 1,11^{ns}	11,16 ± 1,38^{ns}
***C. sorokiniana* (3 μg/mL)**	16,01 ± 0,33*	13,65 ± 1,39***	12,90 ± 1,15***
***D. bardawil* (1 μg/mL)**	15,98 ± 0,34*	12,34 ± 1,20^{ns}	11,54 ± 1,42^{ns}
***D. bardawil* (3 μg/mL)**	16,01 ± 0,29*	13,10 ± 1,54**	12,73 ± 1,28***
Phytoene (1 μg/mL)	15,94 ± 0,39^{ns}	12,59 ± 1,45^{ns}	11,16 ± 1,54^{ns}
Phytoene (3 μg/mL)	15,97 ± 0,35^{ns}	13,34 ± 1,35***	12,53 ± 1,36***
Lutein (1 μg/mL)	16,02 ± 0,28**	12,78 ± 1,38*	11,65 ± 1,56^{ns}
Lutein (3 μg/mL)	15,97 ± 0,35^{ns}	13,46 ± 1,25***	12,79 ± 1,25***

Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. n = 140 worms per condition. Data are presented as mean ± standard deviation (SD). Stars show significant differences between each treatment and control (DMSO): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns: not significant. OP50: *Escherichia coli* OP50; DMSO: dimethylsulfoxide.

some cases, even desirable aesthetic benefits (Meléndez-Martínez et al., 2019). Lutein is a dietary carotenoid present in human skin and is thought to contribute to its antioxidant defense system. Lutein has been shown to provide protection against skin damage caused by UV light in animal studies, and it may have the capacity to absorb visible blue-light wavelengths in the skin (Roberts et al., 2009). A particular study carried out in mice evaluated the effect of a diet supplemented with 10 mg of lutein and 2 mg of zeaxanthin for 12 weeks. It was observed that the supplemented group exhibited improved skin tone clarity, reduced lines and wrinkles, and enhanced skin firmness (elastic recovery) compared to a placebo group fed the same diet without this supplementation (Juturu et al., 2016). Additionally, in a clinical trial with 65 human subjects, consumption of a carotenoid-rich extract of golden tomatoes containing phytoene and phytofluene significantly reduced trans-epidermal water loss (TEWL) in those with elevated TEWL at baseline, suggesting improved skin barrier function. These results were further supported by in vitro gene expression analysis conducted within the same study, where cultured dermal fibroblasts treated with the same extract showed upregulation of genes related to innate immunity, DNA repair, and detoxification pathways (Tarshish et al., 2022). Moreover, a double-blind, placebo-controlled clinical study with a tomato nutrient complex containing lycopene, phytoene, phytofluene, and β-carotene showed significant protection against UVB-induced erythema and inflammation in humans. Specifically, the supplement suppressed the expression of the proinflammatory cytokines IL-6 and TNF-α after UVB exposure and significantly increased the plasma levels of the respective carotenoids (Groten et al., 2019).

Seam cells play a crucial role in the formation of the epidermis and the cuticle (Altun & Hall, 2009; Chisholm & Xu, 2012). The wild-type *C. elegans* at the end of post-embryonic development displays an average of 16 seam cells per lateral side and this number is very robust with minimal variation in the population, thereby offering an easy read-out to assess epidermal development (Katsanos et al., 2017). Seam cell number is more dynamic during post-embryonic development because wild-type nematodes are born with 10 seam cells per lateral side at the L1 stage and these cells divide and differentiate to produce epidermal, neuronal and support cells (Sulston & Horvitz, 1977). Feeding the nematodes with the different carotenoids led to significant differences in seam cell number in wild type in some cases. Phytoene-enriched *C. sorokiniana* (3 μg/mL), phytoene-enriched *D. bardawil* (1 and 3 μg/mL), and pure lutein (1 μg/mL) showed a significant increase in seam cell count compared to the control (1.01-fold higher). Mutants containing a deletion in the *rnt-1* gene have fewer seam cells per lateral side compared to wild-type nematodes due to defective symmetric divisions at L2 stage (Kagoshima et al., 2007; Nimmo et al., 2005). RNT-1 promotes symmetric divisions by suppressing POP-1, a transcription factor downstream of Wnt signaling pathway that specifies fate during asymmetric division (Van der Horst et al., 2019). In the *rnt-1* mutant, lutein at a concentration of 1 μg/mL (average of seam cell number per lateral side: 12.78) and all four samples at a concentration of 3 μg/mL (phytoene-enriched *C. sorokiniana* (13.65), phytoene-enriched *D. bardawil* (13.10), pure phytoene (13.34), and pure lutein (13.46)) showed a significant improvement in seam cell number compared to control ($p < 0.05$). However, it should be emphasized that the effects of *C. sorokiniana* extract cannot be attributed only to lutein. Supplementary Fig. 1 shows that this extract also contains notable amounts of phytoene, which may contribute to the observed biological outcomes. Therefore, the observed outcomes are more likely the result of combined carotenoid activity rather than a single compound. Similarly, *egl-18,* a downstream target of POP-1 in the Wnt signaling pathway, is required for seam cell maintenance and its loss leads to seam cell loss over development (Gorrepati et al., 2013; Koh & Rothman, 2001). In this strain, all the samples receiving compounds dosed at 3 μg/mL showed highly significant differences compared to the control ($p < 0.001$). Among the treatments at 3 μg/mL, phytoene-enriched *C. sorokiniana* extract, pure lutein, phytoene-enriched *D. bardawil* extract, and pure phytoene led to average seam cell number of 12.90, 12.79, 12.73, and 12.53 per lateral side respectively, in comparison to 12 cells in OP50-fed nematodes. In both mutants, all the samples at the highest concentrations tested (3 μg/mL) led to significantly higher number of seam cells compared to the respective samples at 1 μg/mL ($p < 0.01$), suggesting that increasing the carotenoid concentrations may enhance seam cell proliferation. These findings suggest that carotenoids could have beneficial effects on maintaining the epidermal stem cell pool. Maintaining the epidermal stem cell number is critical for tissue homeostasis, as deficiency can lead to structural cuticle defects in worms (Page & Johnstone, 2007) and is necessary for skin repair and remodelling in humans. The mechanisms by which carotenoids exert these effects are not yet fully understood, but several possibilities can be proposed. Based on prior literature, carotenoids may help reinforce cuticle integrity by supporting collagen synthesis or stability, reducing oxidative stress (Bai et al., 2023; Darvin et al., 2022). In addition, they may modulate epidermal signaling such as the Wnt signaling pathway, which is central for seam cell proliferation and maintenance. The improvements observed after carotenoid supplementation in the mutants are compatible with a partial support of Wnt-dependent processes, helping to compensate for gene loss and preserve seam cell numbers. For instance, astaxanthin has been reported to interfere with Wnt/β-catenin signaling in a mammalian model (Kavitha et al., 2013), supporting the idea that carotenoids can modulate this pathway. However, we note that we have not used here direct readouts of the above pathways so future experiments are required to confirm whether these mechanisms operate in *C. elegans*.

Beyond these mechanistic insights, our results also point to possible synergistic interactions. Although the concentrations of phytoene and lutein in the microalgal extracts were lower than in the pure standards, the extracts often showed stronger effects. This suggests that other

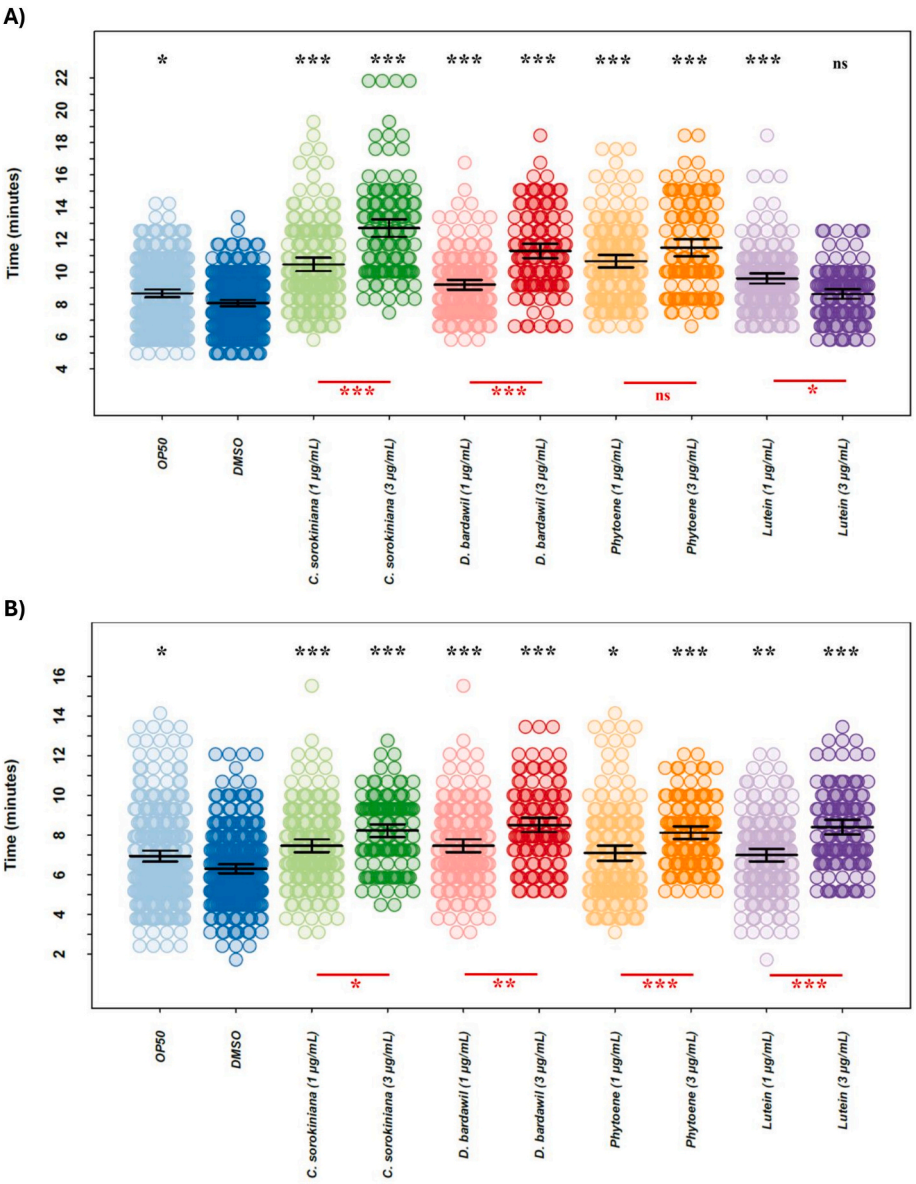

Fig. 2. Time to cuticle rupture in wild-type **(A)** and *egl-18(ga97)* mutant **(B)** animals treated with *C. sorokiniana* and *D. bardawil* extracts and pure phytoene and lutein, exposed to bleaching solution. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. $n = 160$ worms per condition. Black stars show significant differences between each treatment and control (DMSO). The error bars in the figures represent the standard deviation (SD). Among the same compounds, red stars show significant differences between both concentrations evaluated (1 and 3 µg/mL). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns: not significant. OP50: *Escherichia coli* OP50; DMSO: dimethylsulfoxide. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

compounds present in the extracts may contribute alongside carotenoids. In particular, lutein, phytoene, and zeaxanthin may provide overlapping but distinct antioxidant and photoprotective actions, while additional bioactive molecules such as lipids and proteins could help stabilize carotenoids, improve their uptake, or modulate epidermal signaling activity. Together, these interactions could explain the stronger reinforcement of cuticle integrity and seam cell proliferation observed with whole extracts compared to single carotenoids. Similar synergistic effects of carotenoid mixtures and matrix components have also been reported in food extracts and clinical studies (Groten et al., 2019; Tarshish et al., 2022). In addition, combinations of carotenoids and phenolics have shown stronger antioxidant effects than the individual compounds alone (González-Peña et al., 2023). Similarly, studies in humans have demonstrated that mixtures of carotenoids exert greater protective effects than single carotenoids, supporting the idea that whole extracts can be more effective than isolated compounds (Jayedi et al., 2018).

Furthermore, the distinct responses observed between the two microalgal extracts may be related to their specific carotenoid profiles. In particular, *D. bardawil* is characterized by higher levels of zeaxanthin, a carotenoid with potent antioxidant and membrane-stabilizing properties (Różanowska et al., 2021), which may contribute to its stronger protective effects on cuticle integrity. By contrast, *C. sorokiniana* contains relatively higher amounts of lutein and phytoene (Supplementary Fig. 1), which may play a greater role in promoting seam cell proliferation. These compositional differences could therefore account for the differential responses of the two extracts across the mutant strains.

While carotenoids have been described to have positive effect on longevity and stress resistance and can rescue neurodevelopmental defects in *C. elegans* (Ding & Zhao, 2022, 2025; Lashmanova et al., 2015; Lin et al., 2024; Maglioni et al., 2022; Martorell et al., 2020; Morón-Ortiz, Karamalegkos, Mapelli-Brahm, Ezcurra and Meléndez-Martínez,

Table 2
Time for cuticle rupture under bleaching solution treatment in wild-type and *egl-18(ga97)* mutants treated with *C. sorokiniana* and *D. bardawil* extracts and pure phytoene and lutein.

	JR667	MBA290 - *egl-18(ga97)*
OP50	8,67 ± 2,04*	6,94 ± 2,38*
DMSO	8,07 ± 1,66	6,30 ± 1,91
***C. sorokiniana* (1 µg/mL)**	10,46 ± 2,65***	7,47 ± 2,07***
***C. sorokiniana* (3 µg/mL)**	12,71 ± 3,01***	8,23 ± 1,76***
***D. bardawil* (1 µg/mL)**	9,21 ± 1,96***	7,67 ± 2,45***
***D. bardawil* (3 µg/mL)**	11,30 ± 2,45***	8,51 ± 1,98***
Phytoene (1 µg/mL)	10,66 ± 2,46***	7,09 ± 2,43*
Phytoene (3 µg/mL)	11,49 ± 2,89***	8,13 ± 1,71***
Lutein (1 µg/mL)	9,59 ± 2,03***	7,00 ± 2,03**
Lutein (3 µg/mL)	8,63 ± 1,63^{ns}	8,40 ± 2,00***

Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. n = 160 worms per condition. Data are presented as mean ± standard deviation (SD). Stars show significant differences between each treatment and control (DMSO): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns: Not significant. OP50: *Escherichia coli* OP50; DMSO: dimethylsulfoxide.

2024), this represents the first investigation of their effect on the epidermis and cuticle of *C. elegans*. For example, supplementation with 1 µg/mL of phytoene and phytoene-enriched extracts from *C. sorokiniana* and *D. bardawil* have been evaluated in N2 *C. elegans* lifespan. This study concluded that these samples significantly increased life expectancy by 15.5 %, 10.0 %, and 18.6 %, respectively, compared to a DMSO control (Morón-Ortiz, Mapelli-Brahm, & Meléndez-Martínez, 2024). Additionally, Lee et al. (2022) investigated the effect of β-carotene, canthaxanthin, and astaxanthin from the microalgae *Haematococcus lacustris* at 0.5 and 5 µM on the *C. elegans* life expectancy. The investigation found that, regardless of the carotenoid concentration, all of them resulted in a significant increase in lifespan of ~30 % compared to control. Moreover, astaxanthin, a xanthophyll that can be found in high concentrations in the microalgae *Haematococcus pluvialis*, is increasingly recognized for its health and dermatological benefits. This carotenoid has been shown to inhibit collagenases and matrix metalloproteinases, reduce inflammatory mediators, and prevent the induction of ROS in human dermal fibroblasts. These actions contribute to its anti-wrinkle and antioxidant effects (Davinelli et al., 2018). How carotenoids modulate seam cell number remains unknown and a key first step would be to understand though detailed lineage analysis whether the effects are mostly driven by changes in cell proliferation, cell differentiation or both.

The time it takes for chemicals to lead to visible phenotypes in the worm such as paralysis or cuticle rupture has been used as a proxy for cuticular integrity (Loer et al., 2015; Sandhu et al., 2021). The mean time to cuticle rupture of the *egl-18(ga97)* mutant was shorter compared to that of the wild-type strain, as expected. This observation is consistent with the known cuticular defects in the *egl-18(ga97)* mutant, which compromises its structural integrity and increases its susceptibility to external agents such as the bleaching solution (Koh & Rothman, 2001). Enhanced resistance to bleaching-induced cuticle rupture was observed in both the wild type and the mutant, when the nematodes were supplemented with all of the tested samples, except lutein at a concentration of 3 µg/mL in the wild-type strain. The sample that showed the highest bleaching resistance in the wild type was phytoene-enriched *C. sorokiniana* extract at a concentration of 3 µg/mL, with an average time for cuticle rupture of 12.71 min. Both phytoene-enriched microalgae extracts showed significantly better resistance at 3 µg/mL than at 1 µg/mL ($p < 0.001$). In the *egl-18(ga97)* mutant, all tested samples significantly improved cuticle rupture at both concentrations ($p < 0.05$), with the 3 µg/mL concentration showing a highly significant enhancement ($p < 0.01$). The extract that extended the cuticle rupture time the most was the phytoene-enriched *D. bardawil* extract at a concentration of 3 µg/mL, with an average time of 8.51 min. All extracts at 3 µg/mL

Table 3
Percentage of blue-stained animals after Hoechst stain assay in the *bus-19 (e2912)* mutant treated with *C. sorokiniana* and *D. bardawil* extracts and pure phytoene and lutein. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. n = 150 worms per condition. Data are presented as mean ± standard deviation (SD). Stars show significant differences between each treatment and control (DMSO): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns: not significant. OP50: *Escherichia coli* OP50; DMSO: dimethylsulfoxide.

	CB6338 - *bus-19(e2912)*
OP50	16,00 ± 3,00***
DMSO	41,33 ± 1,15
***C. sorokiniana* (1 µg/mL)**	6,67 ± 1,15***
***C. sorokiniana* (3 µg/mL)**	4,67 ± 1,00***
***D. bardawil* (1 µg/mL)**	5,33 ± 1,53***
***D. bardawil* (3 µg/mL)**	6,67 ± 1,53***
Phytoene (1 µg/mL)	4,00 ± 2,52***
Phytoene (3 µg/mL)	5,33 ± 1,53***
Lutein (1 µg/mL)	9,33 ± 0,58***
Lutein (3 µg/mL)	3,33 ± 1,15***

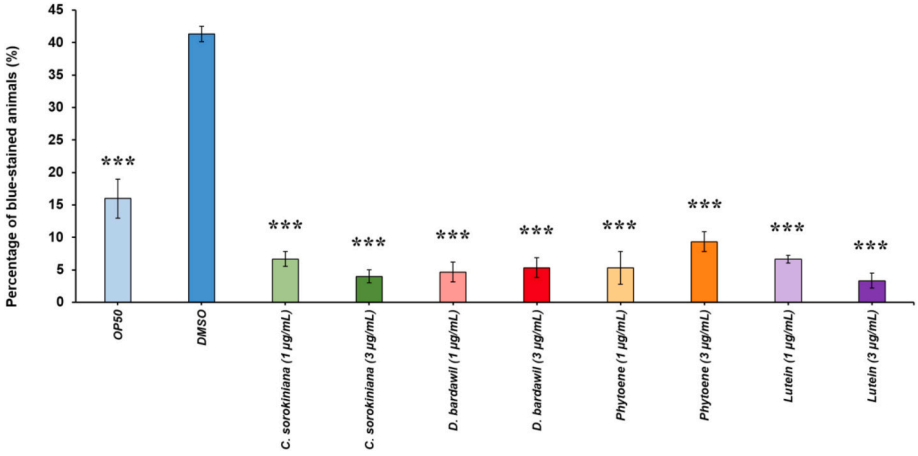

Fig. 3. Percentage of blue-stained animals after Hoechst stain assay in *bus-19* mutants treated with *C. sorokiniana* and *D. bardawil* extracts and pure phytoene and lutein. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. $n = 150$ worms per condition. The error bars in the figures represent the standard deviation (SD). Stars show significant differences between each treatment and control (DMSO): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns: not significant. OP50: *Escherichia coli* OP50; DMSO: dimethylsulfoxide. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

resulted in a longer bleaching time resistance compared to 1 μg/mL, with these differences being statistically significant in all cases ($p < 0.05$). Possible mechanisms for this improvement could be through increased synthesis or decreased degradation of collagen (Sandhu et al., 2021), or the upregulation of proteins or enzymes that play a role in cross-linking collagen fibers, thereby increasing cuticle toughness (Sundaram & Pujol, 2024), hypotheses that need to be tested in future studies. Direct observation of the cuticle through electron microscopy, investigation of its mechanical parameters as well as gene expression and metabolic studies upon carotenoid treatment can help dissect the exact mechanisms at play (Ding & Zhao, 2025; Essmann et al., 2017; Rahimi et al., 2022). It is of note that higher doses of the samples have been shown to enhance resistance to cuticle bleaching in both strains evaluated (except for pure phytoene in the wild-type strain). However, in the wild-type strain, higher doses of lutein (3 μg/mL) have not resulted in significant improvements compared to DMSO, stressing the need to understand the dose-response mechanism in order to determine the optimal concentrations that maximise benefits while minimising risks (Kirchweger et al., 2023). This pattern is consistent with a hormetic dose-response ('U-shaped') response, whereby low concentrations of carotenoids may enhance antioxidant defences or beneficial signaling, while higher concentrations may saturate transport/binding processes, alter membrane microenvironments, or exert pro-oxidant/antagonistic effects that attenuate the net benefit. Biphasic responses of this type have been described for carotenoids and other antioxidants in biological systems. Finally, this study also aimed to assess the effect of carotenoids on cuticle permeability in *C. elegans*. The *bus-19* gene encodes a conserved transmembrane protein, and its deletion can cause more permeable and morphologically defective cuticles compared to the wild type (Yook & Hodgkin, 2007). Therefore, *bus-19* mutants were used to explore whether any treatment could suppress the effects of the *bus-19* mutation and lead to less permeable, healthier cuticle. Cuticle permeability was assessed based on the percentage of worms that presented Hoechst staining post treatment. The results showed that the treatment with all the samples at both concentrations (1 and 3 μg/mL) resulted in a significant reduction of cuticle permeability compared to DMSO control, ranging from 4.43 to 12.41-fold lower. Moreover, all samples tested resulted in lower permeability values compared to OP50, with statistically significant differences observed in phytoene-enriched *D. bardawil* at 1 μg/mL ($p < 0.05$), phytoene-enriched *C. sorokiniana* at 3 μg/mL ($p < 0.05$), and pure lutein at 3 μg/mL ($p < 0.01$). The improvement in permeability after treatment indicates a restoration of the barrier function of the epidermis, which can have a significant impact on animal health. The reduction in Hoechst staining after carotenoid administration is consistent with barrier reinforcement: carotenoids can reduce oxidative stress and promote the maintenance of the cuticle and extracellular matrix, resulting in reduced permeability (Darvin et al., 2022; Sandhu et al., 2021; Sundaram & Pujol, 2024). In *bus-19*, which has a more permeable baseline (Yook & Hodgkin, 2007), the improvement is more evident. The epidermis acts as a first line of defense against pathogens, preventing infection and physical damage in *C. elegans* (Chisholm & Xu, 2012). In addition, it prevents dehydration and uncontrolled exchange of solutes between the internal and external environment. In the context of *C. elegans*, a healthy cuticle is also crucial for growth, survival, and interaction with its environment (Wang et al., 2024).

The findings show that the carotenoids and carotenoid-rich extracts can positively influence seam cell number, enhance resistance to bleaching, and reduce cuticle permeability in *C. elegans*. While the specific mechanisms underlying these effects remain to be elucidated, our results are of translational relevance, as epidermal proliferation and barrier reinforcement in nematodes parallel processes of epidermal renewal and barrier function in human skin. This provides promising evidence supporting the role of carotenoids in maintaining epidermal integrity and cuticle function, with potential implications for wound repair, hydration, and broader implications for future developments in the field of dermocosmetics.

CRediT authorship contribution statement

Ángeles Morón-Ortiz: Writing – original draft, Investigation, Formal analysis. **Mar Ferrando-Marco:** Investigation, Formal analysis. **Antonio León-Vaz:** Writing – original draft, Investigation, Formal analysis. **Rosa León:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Paula Mapelli-Brahm:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Michalis Barkoulas:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Meléndez Martínez, Antonio Jesús:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.147022.

Data availability

No data was used for the research described in the article.

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