An innovative extract of the microalga *Haematococcus salinus Dunal*. to fight Glyc-Aging™ and protect the skin from intense solar irradiation.

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Abstract

Background: Glycation and the resulting buildup of Advanced Glycation End products (AGEs) (Glyc-Aging™), driven *inter alia* by solar radiation, are recognized as key contributors to skin aging.

Haematococcus salinus Dunal. is a halophile microalga, adapted to intense solar radiation through carotenoid production. A supercritical CO_2 extract of this alga, containing the colorless carotenoids phytoene and phytofluene, captures the alga's natural adaptation to protect skin from photoaging and Glyc-aging™ through anti-glycative and anti-inflammatory properties.

Methods: Normal human skin explants were treated with extract over 10 days. AGE receptor (RAGE), glyoxalase-1 and key inflammation regulator NRF2 were quantified by immunohistochemistry. IL6 and IL8 were measured by ELISA. Glycation was induced using methylglyoxal (MG), and AGE N-epsilon-(carboxymethyl)lysine (CML) was quantified by immunohistochemistry.

A double-blind, placebo-controlled clinical trial under high solar exposure (peak summer beachgoers) assessed: glycation (AGE Reader®); anti-inflammatory effects (Doppler laser microcirculation with histamine stimulation); red, UV spots (VISIA-CA); wrinkling, roughness (AEVA-HE); and biomechanics (Cutometer®).

Results: *Ex vivo*, the extract strongly reduced MG-induced CML formation, reduced RAGE levels, significantly reduced IL6 and IL8, and increased NRF2.

Clinically, under intense solar exposure, vs. placebo, the extract: significantly reduced glycation; reduced inflammation (reduced red spots; increased resiliency to histamine challenge), UV spotting, skin roughness; improved skin elasticity and firmness; and strongly

improved wrinkling – both preventing solar-induced damage and bringing a net improvement vs. D0.

Conclusion: These results demonstrate the value of this extract, containing the colorless carotenoids phytoene and phytofluene, as an antiglycative, anti-inflammatory, and anti-aging active, including in high solar irradiation contexts.

Keywords: Aging; Glycation; Inflammation; Carotenoids; Microalga.

Introduction.

Aging may be defined as the progressive accumulation of damage to living tissue over an organism's lifetime. Skin ages both through internal processes and due to external stressors, including sunlight, pollution, and smoking - leading to structural changes affecting its appearance and its biological functions. Aged skin shows a loss of elasticity, wrinkling, dryness, reduced thickness/atrophy, reduced cell proliferation, and accumulated cellular senescence [1].

Accumulation of Advanced Glycation End products (AGEs) in tissues is now recognized as a key contributor to skin aging [2]. AGEs are formed through glycation, a complex process involving a spontaneous nonenzymatic reaction between reducing sugars and proteins, lipids, or nucleic acids, where the sugar's carbonyl groups react, for instance, with free amino groups on proteins – eventually forming Amadori ketoamines. These subsequently undergo irreversible oxidation, polymerization, dehydration, and cross-linking reactions to generate AGEs [3]. Carboxymethyl-lysine (CML), carboxyethyl-lysine, and fructose-lysine are some of the most commonly found AGEs in skin. Receptors for AGEs (RAGEs) are expressed in the epidermis and dermis, and their expression, as well as AGE accumulation, has been shown to be higher in sun-exposed areas of the skin as compared with sun-protected areas [4] – indicating that solar irradiation likely accelerates this process.

Accumulation of AGEs in the skin has also been observed in diabetes and during baseline chronological aging. Proteins with a slow turnover rate such as collagens I and IV or fibronectin are primary targets of glycation in the skin. AGEs generated by smoking or ingested from diet may also participate in skin aging [5–7].

Glycation of biological molecules can hamper their biomechanical and functional properties, through modifications of protein conformation and solubility, enzyme–substrate interactions, protein–DNA interactions, protein–protein interactions, DNA regulation, and epigenetic modulation, thus interfering with numerous physiological functions of the targets of glycation [8]. For example, glycation and cross-linking of collagen or elastin can lead to increased ECM and tissue stiffness, and also raises resistance to removal by matrix metalloproteinases (MMPs) [9]. RAGE activation has been shown to result in inflammatory and immune responses, increased MMP production, impaired cell proliferation, excessive melanogenesis, and altered gene expression [10–13]. All of these processes can potentially drive skin aging.

Biological detoxification of AGEs employs the glyoxalase enzymatic pathway, involving the enzyme glyoxalase-1 (Glo-1), whose main substrate is methylglyoxal (MG), a widely recognized initiator of glycation formed in animal tissues as a byproduct of glycolysis [14–17]. However, in light of the persistent AGE-driven tissue aging phenomena recorded in the literature, it seems that these mechanisms are, alone, insufficient to manage glycation and its effects over a lifetime.

Several strategies for prevention or correction of AGE accumulation have been put forward, including inhibiting AGE formation; removing (degrading) existing AGEs; and antagonizing AGE/RAGE-initiated signaling. Some synthetic AGE inhibitors have been proposed, but so far their efficacies and/or safety profiles have been generally unsatisfactory. Natural compounds including mainly phenolics, but also oligo- and polysaccharides, carotenoids (e.g., β-carotene), and unsaturated fatty acids have been reported to possess antiglycating activity [18]. Inhibiting AGE formation using microalgal extracts, in particular, offers some novelty compared to most botanicals, in that the active compounds involved are generally not phenolic compounds. Instead, a wide range of antioxidant compounds is produced in microalgae, including carotenoids and polyunsaturated fatty acids, such as linoleic acid, arachidonic acid, and eicosapentaenoic acid [19].

Haematococcus salinus Dunal. is a halophile unicellular microalga found in high-salinity environments (salt beds and lakes, evaporation ponds) [20]. This alga adapts to high solar irradiation by producing β-carotene as well as its precursors, the colorless carotenoids phytoene and phytofluene [21]. *Haematococcus salinus Dunal*. also contains ω-3 unsaturated

eicosapentaenoic acid (EPA), generally not found in terrestrial carotenogenic plants. EPA, among other unsaturated fatty acids, has been shown to possess antiglycation properties [22]. Some carotenoids, such as lutein, β-carotene, and astaxanthin, have been shown to have antiglycative power [23]. However, the use of most carotenoids in topical cosmetic formulations remains problematic due to the usually intense color imparted by these compounds to the formula as well as to the skin. The colorless carotenoids phytoene and phytofluene, however, do not have this issue. Phytoene and phytofluene are the biosynthetic precursors for all colored carotenoids and, due to their shorter conjugated C=C double-bond chromophores, absorb light in the UV range and not in the visible range – making them 'colorless'. As key precursors, these colorless carotenoids are found at some level in most carotenogenic organisms, including *Haematococcus salinus Dunal*. (Figure 1). These carotenoids have demonstrated properties useful in health, nutritional, and cosmetic applications, including offering a measure of protection against UV and oxidative damage leading to premature aging and other disorders. The colorless carotenoids have been shown to have antioxidative, anti-inflammatory, and DNA protection activities [24]. We thus hypothesized that phytoene and phytofluene may hold promise for protection from glycation-induced aging and solar damage in skin.

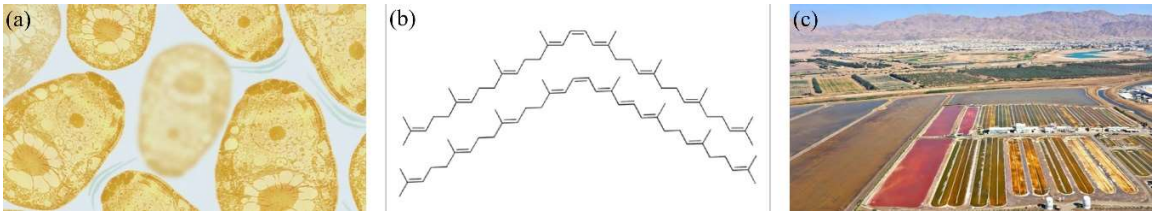

Figure 1. *(a):* Haematococcus salinus Dunal*., illustrative microscopy image; (b): phytoene (top) and phytofluene (bottom);* Haematococcus salinus Dunal*. cultivation ponds (southern Israel) showing a range of colors produced by the alga under various conditions.*

We present here results showing that a hydrophobic extract of *Haematococcus salinus Dunal*., standardized in phytoene and phytofluene, can be effectively used as an active ingredient for the protection of skin against some effects of solar exposure, and that this same extract mitigates glycation and its associated effects on skin, including inflammation and the appearance of signs of aging (wrinkles), by addressing all three antiglycative strategies:

inhibiting AGE formation; removing / detoxifying formed AGEs; and reducing RAGE signaling.

Materials and Methods.

Extract: The studies described herein employed a hydrophobic extract of *Haematococcus salinus Dunal*. (IBR-Solage®, IFF - Lucas Meyer Cosmetics). After harvesting, the alga is dried and extracted with supercritical CO_2, and the resulting oleoresin is taken up in a carrier oil and further purified to remove colored components, retaining the colorless carotenoids. The final extract in jojoba oil is standardized for phytoene and phytofluene content.

Anti-glycation effect of the extract on human skin explants:

This study was performed on human skin tissue obtained from surgical residues in full respect of the Declaration of Helsinki and Article L.1243-4 of the French Public Health Code. Normal human skin explants from a 34-year-old woman were incubated in survival culture medium at 37°C, 5% CO_2 in a humidified atmosphere. A group of explants were left untreated, while another was treated topically with 0.5% extract in jojoba oil on days 3, 4, 5, and 7. Half of each group was challenged with 500 µM methylglyoxal (via the culture medium) from Day 4 so as to induce glycation, while the other half remained unchallenged. After day 10, tissue samples were immunostained for glycation marker N-epsilon-(carboxymethyl)lysine (CML), with a monoclonal CML antibody using a Vectastain avidin/biotin Kit Vector amplifier system, and revealed by VIP. Similarly, immunostaining for RAGE was carried out using a polyclonal anti-RAGE antibody, and revealed by Alexa Fluor 488; nuclei were poststained using propidium iodide. Finally, immunostaining for Glo-1 was carried out with a monoclonal anti-Glo-1 antibody using a Vectastain Kit Vector avidin/biotin amplifier system revealed by VIP. Image analysis was used to quantify the staining intensity.

Anti-inflammatory effect of the extract on human skin explants:

This study was performed on human skin tissue obtained from surgical residues, in full respect of the Declaration of Helsinki and Article L.1243-4 of the French Public Health Code.

Skin biopsies were incubated at 37°C, 5% CO_2 in a humidified atmosphere in complete DMEM. Topical application of the extract at 0.5% in jojoba oil or Jojoba oil alone (control) were carried out over 24 hours. At the end of the incubation period, supernatants were collected and IL6 and IL8 released from the explants were quantified by ELISA. Tissue samples were frozen, and NRF2 was specifically labeled with an NRF2 antibody and revealed with a secondary antibody. Microscopic images were recorded, and the NRF2 signal was quantified by image analysis. Data were submitted to the two-way paired Student t-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Clinical evaluation of protective and anti-aging effects under intense solar exposure:

1% *Haematococcus salinus Dunal*. in a simple gel-cream formulation was used in a double-blind, randomized split-face placebo-controlled trial. The 25 female volunteers, aged 35-60, with Fitzpatrick skin phototype II-IV, and showing signs of aging (wrinkles, fine lines), were selected based on expected daily intense solar exposure over the course of the 56-day study (specifically, beachgoing or similar activities), which was carried out during the peak summer months. The skin glycation status was assessed using an AGE Reader® (Diagnoptics Technologies B.V, The Netherlands), based on skin autofluorescence. The anti-inflammatory effect was evaluated via Doppler laser flowmetry measurement of skin microcirculation (Periflux LDPM PF5000 with PF 5010 laser channel, Perimed, Sweden). Histamine, known to increase capillary permeability and vasodilation and therefore recognized as a reliable model to assess cutaneous susceptibility to inflammation, was dispensed by iontophoresis (Perilont, Perimed, Sweden). Onset time was calculated as the time between the start of the histamine application and the start of blood flow increase. Maximal blood flow increase was also recorded. The effect on red and UV spots was evaluated by cross-polarized image analysis (VISIA-CA, Canfield, USA). The effect on wrinkling and skin roughness was evaluated by 3D image analysis (AEVA-HE, Eotech, France). Finally, the effect on skin biomechanics was evaluated using a Cutometer® (Courage & Khazaka, Germany). Resulting data and percentage variations were submitted to the Paired Student's t-Test or the Wilcoxon test (*$p<0.05$).

Results.

Anti-glycation effect in skin explants:

As expected, methylglyoxal stimulation gave rise to a significant increase in glycation (CML levels) (+51%, *** $p < 0.001$). Treatment with *Haematococcus salinus Dunal*. extract results in a strong anti-glycation effect (Figure 2), both in unchallenged explants (*** $p < 0.001$) and in explants challenged with methylglyoxal (*** $p < 0.001$) – most pronounced in the latter (up to -68%).

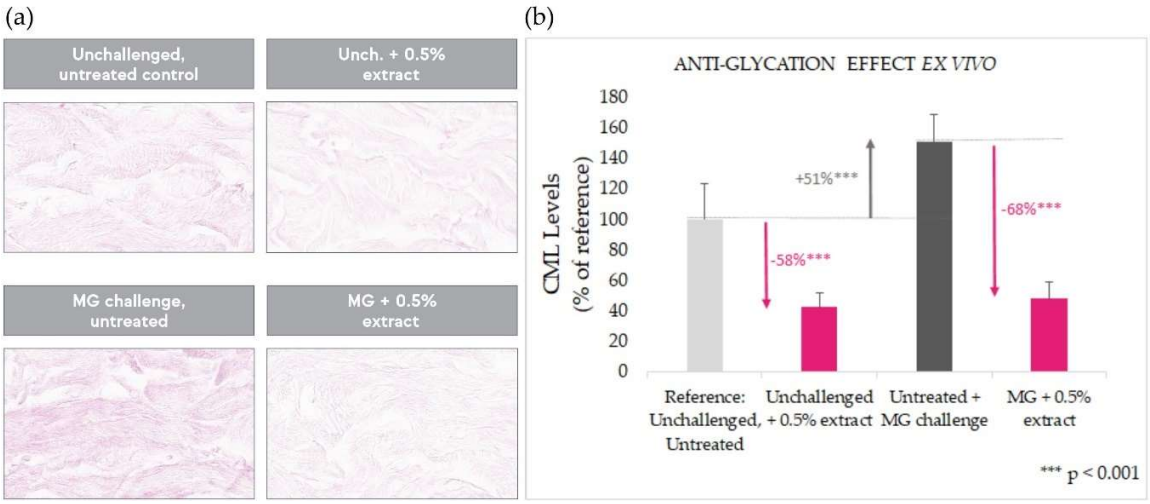

Figure 2: anti-glycation effect. *(a) illustrative images; (b) image analysis results.*

Treatment with the *Haematococcus salinus Dunal*. extract also caused a significant reduction in RAGE and glyoxalase-1 (40% and 24% respectively, * $p < 0.05$), corroborating a reduced glycation state following treatment with the extract (Figure 3).

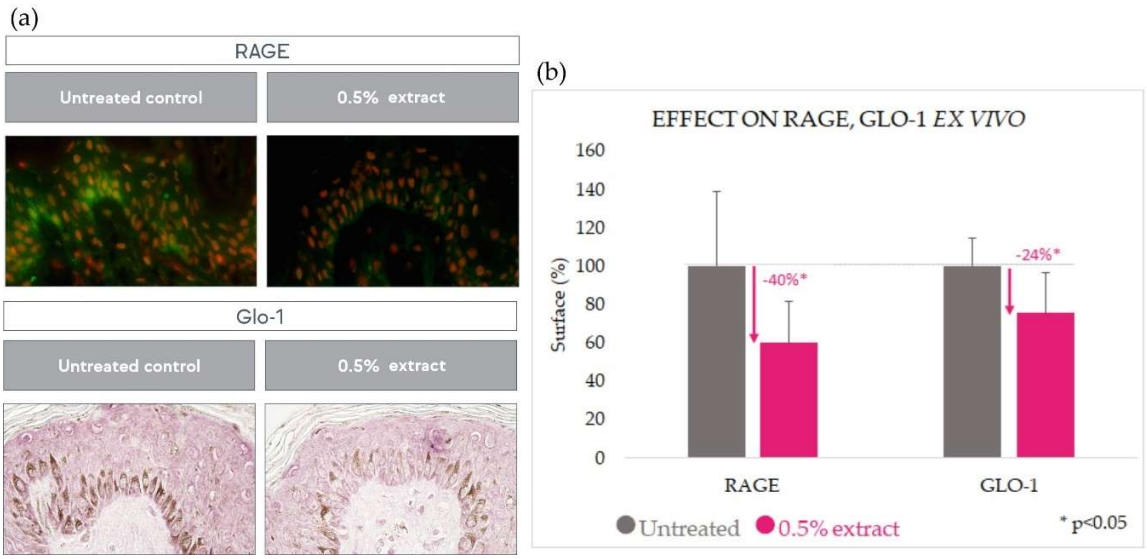

Figure 3. *Reduction of RAGE and Glo-1 in skin explants treated with the* Haematococcus salinus Dunal. *extract. (a): illustrative images. (b): image analysis quantitation results.*

<u>Anti-inflammatory effect in skin explants:</u>

Treatment of skin explants with 0.5% *Haematococcus salinus Dunal*. extract resulted in significant reductions in levels of inflammatory interleukins IL6 and IL8 (by 26% and 45% respectively, * $p < 0.05$; Figure 4), as well as significant enhancement of antioxidant/anti-inflammatory regulator NRF2 (by 19%, $p < 0.001$).

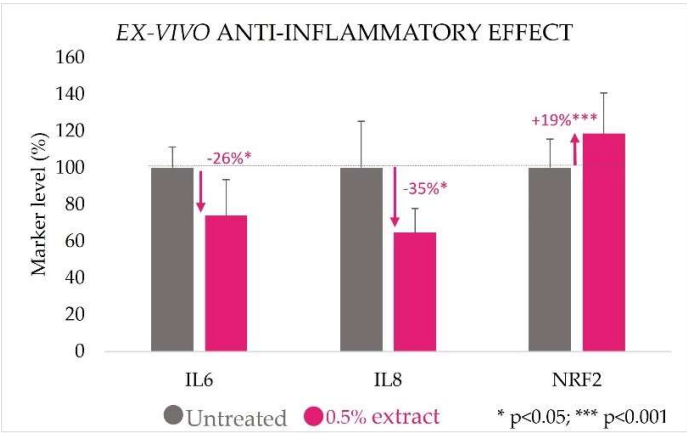

Figure 4: **Ex-vivo *anti-inflammatory effect***: *reduced interleukins 6 and 8, and increased NRF2, upon treatment with the* Haematococcus salinus Dunal. *extract compared with untreated baselines.*

Clinical evaluation of protective effects under intense solar irradiation:

Antiglycation effect: The AGE reader data confirms the indication given by the *ex-vivo* model results detailed above, with a statistically significant reduction in glycation scores with 1% *Haematococcus salinus Dunal*. extract in formulation, both at D28 and D56 (and with * $p < 0.05$ vs. D0 and vs. placebo; Figure 5). The difference in AGE scores for the placebo at D28 or D56 vs. D0 was not statistically significant.

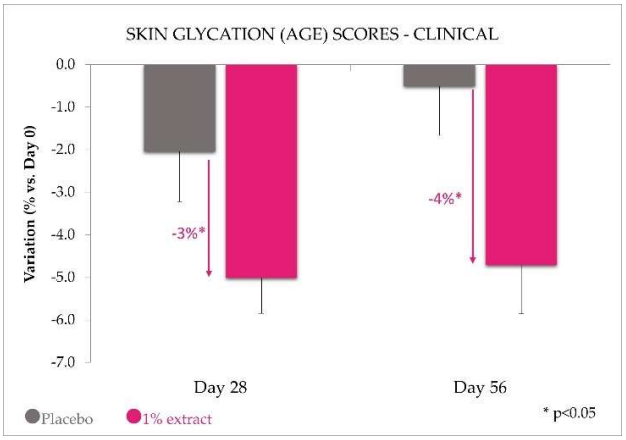

***Figure 5: anti-glycation effect* in vivo.**

Anti-inflammatory effect: After 56 days of treatment, 1% *Haematococcus salinus Dunal*. extract in formulation produced significant improvements in the skin's resilience and its capacity to fight inflammation-inducing provocations, as manifested in a strong reduction of induced microcirculation upon stimulation with histamine (Figure 6; over 35% increase in reaction onset time and 14% reduction in the amplitude of induced microcirculation, * $p <$ 0.05).

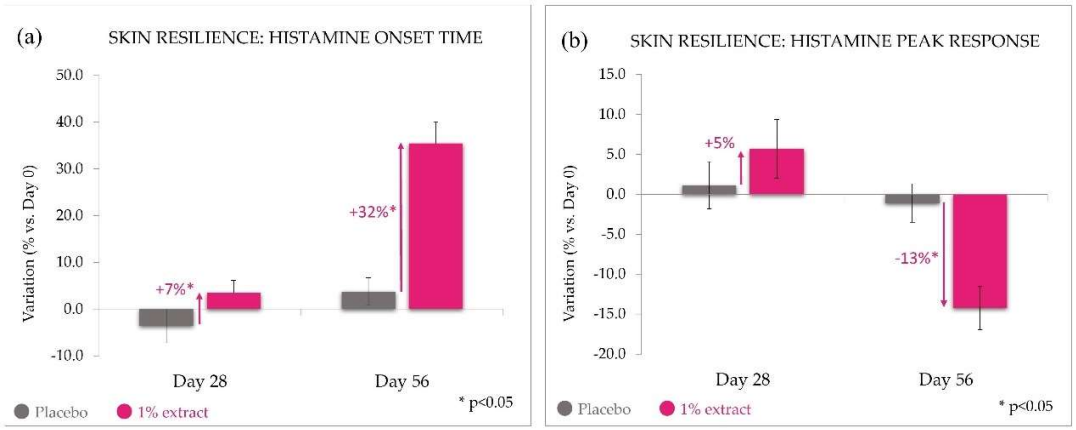

***Figure 6: skin resilience improvement* in vivo.** *(a): onset time of reaction to histamine. (b): peak response intensity. Standard deviation bars were scaled by a factor of 10 for readability.*

At the same time, 1% *Haematococcus salinus Dunal*. extract in formula showed a strong advantage over placebo in red spots counts and areas (Figure 7; -26% and -20% respectively, * $p < 0.05$). While the placebo exhibits the significant increase in red spots expected under intense solar exposure, the active product negates and even reverses this effect, leading to a *decrease* vs. D0. Some of these effects can already be observed at D28, albeit with a lower magnitude and statistical significance.

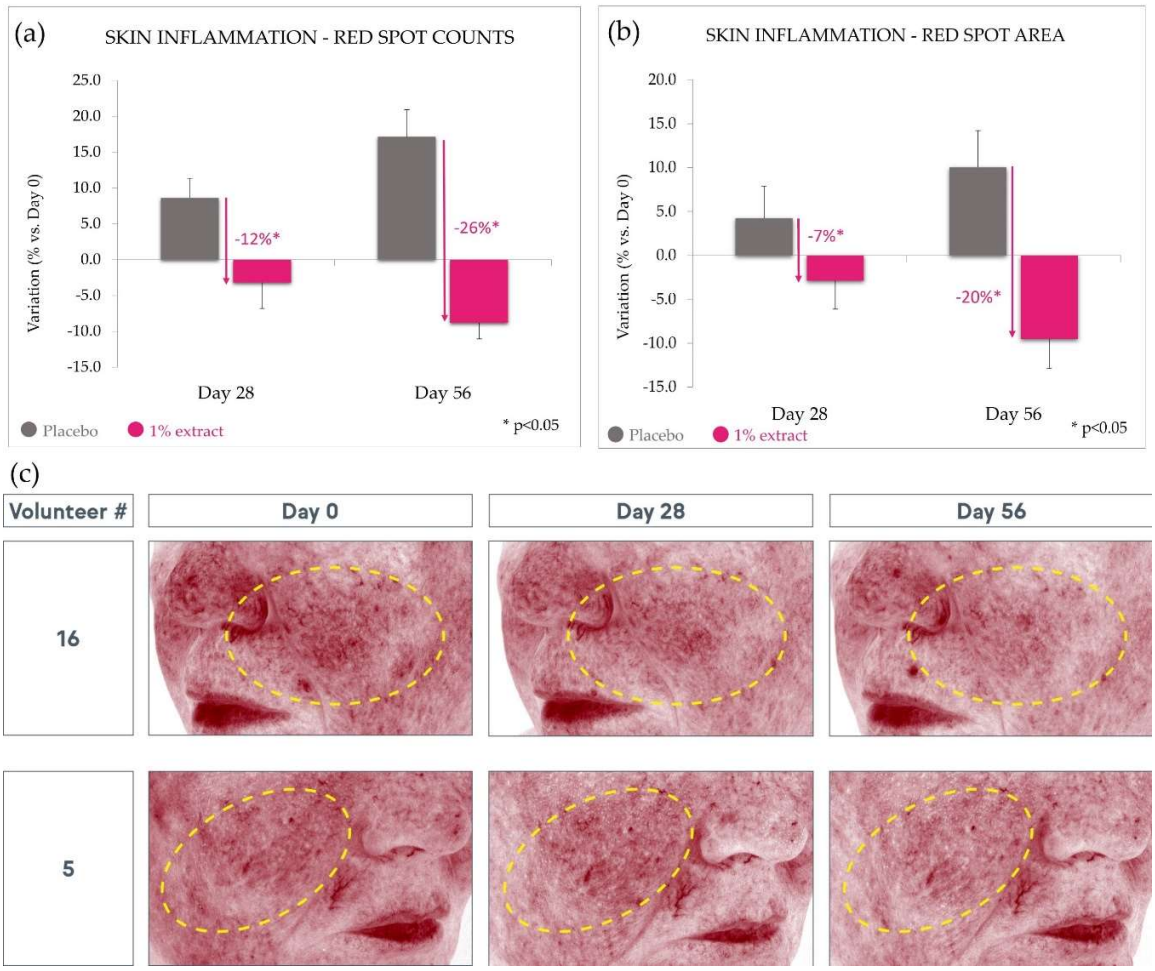

Figure 7: red spots reduction. (a): red spot count reduction; (b): red spot area reduction. (c): illustrative photographs. Standard deviation bars were scaled by a factor of 10 for readability.

Anti-aging effect: Crucially, after 56 days' use, 1% *Haematococcus salinus Dunal*. extract displayed a strong anti-aging effect, as manifested in a significant reduction of wrinkle counts and volume (Figure 8; respectively ca. 32% and 35% vs. placebo, * $p < 0.05$). These effects are also observable at D28 with lower magnitude and statistical significance. It is worth noting that in both cases, a worsening in wrinkling parameters is observed with the placebo product, consistent with the study's high solar exposure conditions. The active product not only negates this effect, but reverses it, producing significant improvements vs. the initial state.

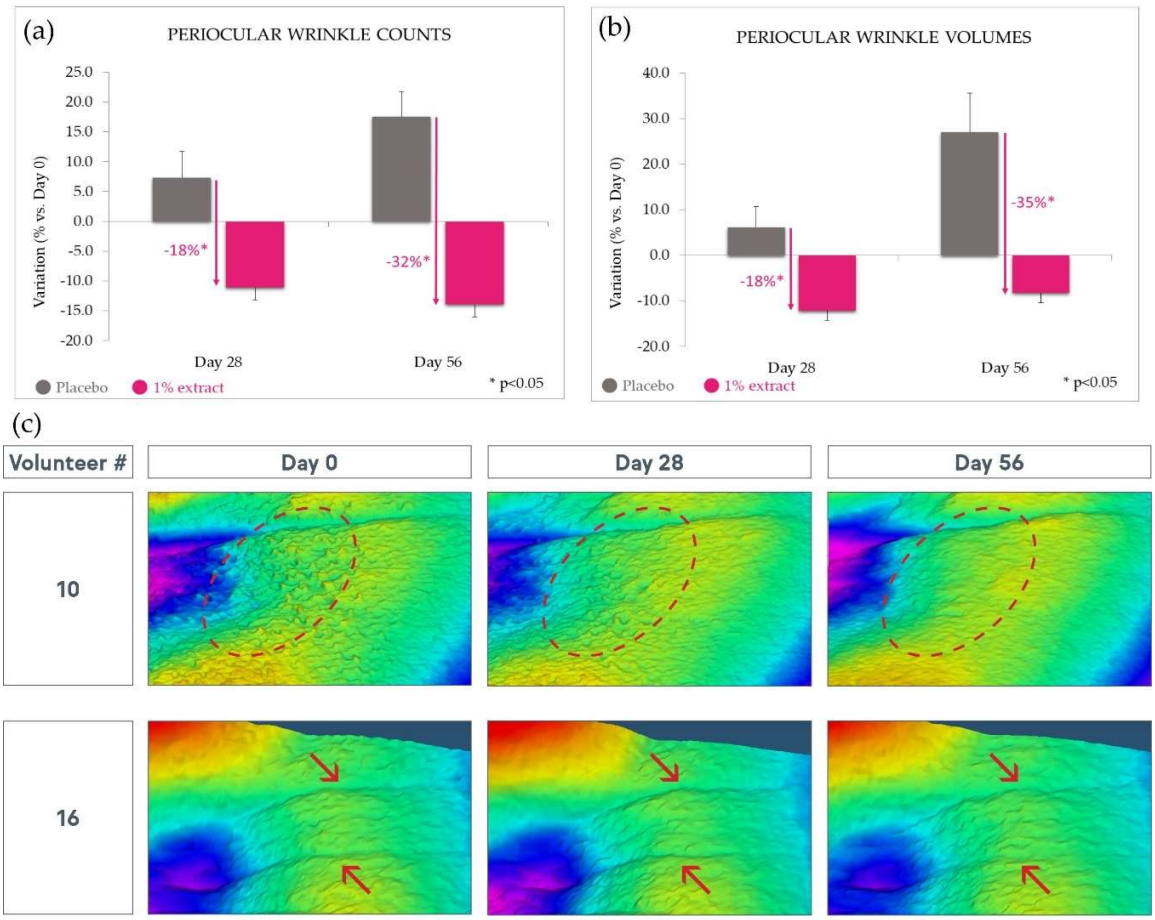

Figure 8: anti-aging effect. (a): wrinkle count reduction; (b): wrinkle volume reduction; (c): illustrative images; circles and arrows emphasize features of interest (skin roughness and wrinkles, respectively). Standard deviation bars were scaled by a factor of 10 for readability.

Effect on UV spots: After 56 days of treatment, the UV spot count shows a reduction with 1% *Haematococcus salinus Dunal*. extract in formula compared to placebo, with statistical significance vs. D0 and placebo (-7% vs. placebo at D56, * $p < 0.05$; Figure 9). Note that as expected from solar exposure, the UV spot count recorded with the placebo group increases over the course of the study. Here again, the active product negates and even reverses this trend.

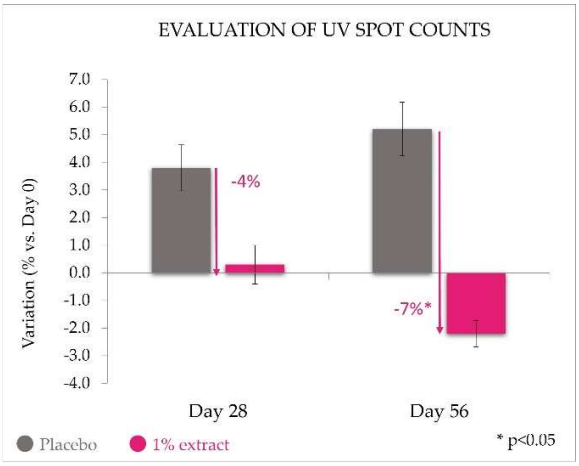

Figure 9: UV spot count reduction. Standard deviation bars were scaled by a factor of 10 for readability.

Effect on skin biomechanics: Finally, the Cutometer data show improvements in skin firmness and skin elasticity with 1% *Haematococcus salinus Dunal*. in formulation after 56 days of use (7% and 14% advantage respectively vs. placebo, with * $p < 0.05$ vs. D0 and vs. placebo; Figure 10). At D28, the effect on skin firmness is nonsignificant vs. placebo, while the effect on elasticity is already detectable, albeit with a smaller break vs. placebo.

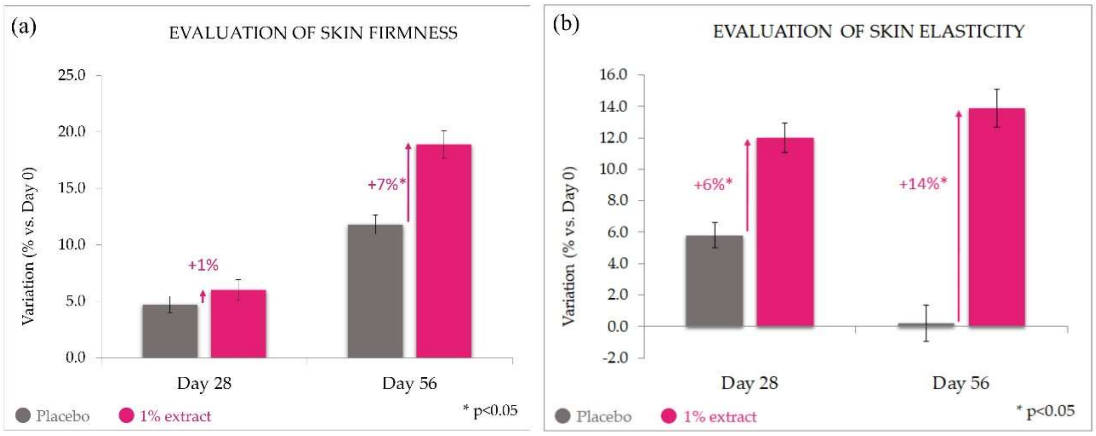

Figure 10: effect on skin biomechanics. (a): skin firmness; (b): skin elasticity. Standard deviation bars were scaled by a factor of 10 for readability.

Discussion. The studies described above indicate that this *Haematococcus salinus Dunal*. extract possesses antiglycation properties, starting with the ability to reduce AGE formation, demonstrated as strongly reduced levels of the glycation marker CML upon treatment with the extract, with and without stimulation of glycation by methylglyoxal, as well as the ability to damp down Age/RAGE signaling, (demonstrated as reduced RAGE levels) and enhance detoxification/removal of formed AGEs (demonstrated via reduced Glo-1).

Additionally, it was shown that the extract possesses anti-inflammatory properties, shown as reductions in interleukins IL6 and IL8, and increased inflammation regulator NRF2 – demonstrating the extract's ability to enhance the skin's adaptation to the downstream effects of glycation and glycation-inducing challenges.

These indications were borne out in a clinical trial under intense solar exposure, where, vs. placebo, treatment with 1% *Haematococcus salinus Dunal.* extract in formulation: reduced glycation; strengthened the skin's resilience to inflammation-inducing insult (represented here by histamine); and strongly reduced redness and wrinkles.

Notably, while the worsening in red spot and wrinkling parameters expected under these high solar irradiation conditions was clearly observed with placebo, the active product not only negated this effect (preventing photodamage), but reversed it, delivering significant improvements vs. D0 in spite of the solar exposure.

Conclusion. The *Haematococcus salinus Dunal*. extract presented above was shown to strongly reduce glycation and inflammation in skin explants, addressing three antiglycative axes: inhibiting AGE formation; removing / detoxifying formed AGEs; and reducing RAGE signaling.

This was confirmed in a clinical trial under intense solar exposure, where the extract reduced glycation, strengthened skin resilience to irritation, and reduced skin redness and wrinkles.

Taken together, these results demonstrate the value of this *Haematococcus salinus Dunal*. extract as an active ingredient in cosmetic applications aimed at protecting the skin from damage and premature aging, including under intense solar exposure conditions.

Conflict of Interest Statement. None.

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