

Blue Beauty: Revealing Bioactive Peptides from Aquaculture Sidestreams for Sustainable Cosmetics

Lorenzo Dondero

University of Genoa

Francesca Tardanico

University of Genoa

Erica Lertora

Angel Consulting S.r.l

Matteo Zanotti Russo

Angel Consulting S.r.l

Federica Robino

Angel Consulting S.r.l

Matteo Sergiampietri

Angel Consulting S.r.l

Mercedes Alonso Martinez

ANFACO-CECOPESCA

Rebeca Vazquez Sobrado

ANFACO-CECOPESCA

Silvia Letasiova

MatTek In Vitro Life Science Laboratories

Jan Markus

MatTek In Vitro Life Science Laboratories

Kjersti Lian

Nofima

Marte Jenssen

Nofima

Mario Marini

University of Milano-Bicocca

Maddalena Collini

University of Milano-Bicocca

Giuseppe Chirico

University of Milano-Bicocca

Sara Ferrando

University of Genoa

Giacomo Rosa

University of Genoa

Giulia De Negri Atanasio

giulia.denegri@edu.unige.it

University of Genoa

Elena Grasselli

University of Genoa

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Abstract

The valorization of aquaculture side-streams represents a pivotal strategy for the circular blue economy, providing high-value biomolecules for cosmetic applications. This study characterizes a < 3 kDa protein fraction, referred to as Marine Hydrolyzed Peptides (MHP), derived from sea bream and sea bass side-streams, and evaluates its cosmetic-relevant biological activity in human skin models. Biochemical analysis confirmed a high hydroxyproline content, corresponding to 42% of the total protein fraction, together with intrinsic antioxidant potential. In human keratinocytes (HaCaT cells), MHP was tested as an aqueous solution (S-MHP) and exhibited cytoprotective and restorative effects against H₂O₂-induced oxidative stress and UVA radiation. Subsequently, MHP was incorporated into a cosmetic formulation (F-MHP) and applied to a reconstructed 3D human skin model for 24 h. A single topical application of F-MHP modulated extracellular matrix-related genes associated with structural organization, elasticity and hydration. Histological and two-photon microscopy analyses revealed an improved matrix fiber organization. Overall, this study demonstrates the feasibility of transforming aquaculture side-streams into biologically validated marine-derived cosmetic actives and establishes a transferable human-relevant workflow for the biological validation of sustainable marine cosmetic ingredients, supporting their translation from circular bioeconomy resources to formulated cosmetic products.

1. Introduction

The blue economy framework increasingly promotes the valorization of fish side streams as sustainable sources of high-value bioactive compounds, moving beyond traditional by-product management toward a circular economy model (Venugopal et al. 2023). Within this strategic context, the H2020 EcoeFISHent project aims to establish a sustainable territorial cluster to demonstrate the upcycling of fish-processing side streams into high-added-value products (EU-CORDIS 2021). Marine fish processing currently generates significant amounts of by-products, including heads, skins, bones and viscera, which represent a rich reservoir of proteins, lipids, vitamins, minerals and other bioactive compounds (Välimaa et al. 2019; Orlandi et al. 2023). However, recovering bioactive molecules from marine side streams is only the first step. The major challenge is demonstrating that these fractions possess sufficient biological efficacy and technological suitability to become viable cosmetic active ingredients. Addressing this challenge requires experimental strategies capable of progressively validating ingredients, from the identification of biologically active concentrations to the assessment of their efficacy after incorporation into cosmetic formulations.

Recently, enzymatic hydrolysis and fractionation of pre-processed side streams from sea bream and sea bass have enabled the production of protein hydrolysate fractions with distinct chemical profiles, molecular weight distributions, techno-functional properties and biological activities (Babu and Kudre 2026). These marine-derived fractions may be particularly relevant for skin-related applications, as low-molecular-weight peptides can interact with cellular pathways involved in stress response, tissue repair, and extracellular matrix homeostasis (Dondero et al. 2025b).

Human skin is constantly exposed to both intrinsic and extrinsic stressors that disrupt cellular homeostasis and contribute to skin ageing. Intrinsic ageing is a physiological process associated with the progressive decline of cellular and extracellular matrix functions, whereas extrinsic ageing is mainly driven by environmental factors, including solar radiation, pollution and other pro-oxidant stimuli. Despite their different origins, both intrinsic and extrinsic ageing share oxidative stress as a key underlying mechanism, leading to the generation of reactive oxygen species (ROS) (Tang et al. 2025), including hydrogen peroxide (H_2O_2), superoxide anion and singlet oxygen. Excessive ROS production can damage DNA, lipids and proteins in keratinocytes and fibroblasts, thereby impairing epidermal barrier function, dermal matrix homeostasis, and skin regenerative capacity. Strategies aimed at protecting the skin from oxidative damage include both the use of sunscreens, which reduce UV-induced ROS generation, and cosmetic formulations containing antioxidant molecules, which help counteract oxidative stress and support skin homeostasis (Chaiprasongsuk and Panich 2022).

Antioxidants have been reported to help counteract ROS-mediated damage and attenuate some of the cellular and tissue alterations associated with skin ageing (Podda and Grundmann-Kollmann 2001; Masaki 2010). In this context, low-molecular-weight peptides (< 3 kDa) have attracted growing interest as cosmetic ingredients with potential protective, regenerative and anti-ageing properties (Sibilla et al. 2015). However, the cosmetic potential of these ingredients cannot be inferred solely from their biochemical composition or antioxidant capacity but requires experimental validation in human-relevant models able to capture both cellular stress responses and tissue-level effects.

In the European Union, animal testing for cosmetic products and cosmetic ingredients is prohibited (Chapter V, art. 18 of Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products) (Regulation (EC) No 1223/2009 2009), making human-relevant in vitro approaches essential for cosmetic assessment (Pistollato et al. 2021). In this context, OECD-validated Test Guidelines based on reconstructed human epidermis models have established robust Standard Operating Procedures for safety endpoints, such as skin irritation and corrosion, thereby paving the way for the broader use of reconstructed human skin models in cosmetic research (OECD 2025; OECD 2026a). However, efficacy testing of cosmetic actives is not yet covered by specific regulatory NAMs or harmonized OECD guidelines and therefore relies on the scientific robustness of the experimental design, the biological relevance of the selected models and the mechanistic consistency of the endpoints investigated (Lautan et al. 2026).

Our previous work established a stepwise in vitro strategy combining 2D and 3D models to evaluate marine-derived cosmetic ingredients (Dondero et al. 2025a). While 2D cellular assays proved suitable for the preliminary screening of active-ingredient biocompatibility and biological efficacy, allowing the identification of the concentrations to be incorporated into the cosmetic formulation, reconstructed full-thickness 3D human skin models provided a more physiologically relevant platform for evaluating the efficacy of the formulated product under conditions that more closely resemble topical application. However, the biological performance of marine protein hydrolysates depends on their source, processing, and formulation (Grasso et al. 2023). The low-molecular-weight protein fraction investigated

in the present study, obtained from sea bream and sea bass aquaculture side-streams, had previously demonstrated the ability to enhance HaCaT keratinocytes viability and promote wound closure, supporting its potential for skin-related cosmetic applications.

Building on this methodological framework and on the preliminary biochemical and biological characterization of a low-molecular-weight (< 3 kDa) protein hydrolysate obtained from sea bream and sea bass aquaculture side streams, the present study established a stepwise human-relevant validation strategy for its translation into a cosmetic active ingredient. Following preliminary biochemical analyses, the hydrolysate was evaluated as an aqueous solution (S-MHP) in HaCaT keratinocytes and RAW 264.7 murine macrophages to identify biologically active concentrations suitable for cosmetic formulation based on their antioxidant, photoprotective and immunomodulatory activities. Since the incorporation of bioactive molecules into delivery systems can markedly influence their stability, release kinetics and biological performance (Venkataramani et al. 2020; Hu and He 2021; De Negri Atanasio et al. 2022; Campardelli et al. 2023), the selected concentrations were subsequently incorporated into a cosmetic cream (F-MHP) and evaluated in a reconstructed full-thickness human skin model. This model was used to determine whether the biological efficacy observed for the active ingredient was retained after formulation and translated into anti-ageing efficacy under physiologically relevant conditions by assessing extracellular matrix-related gene expression, dermal architecture and collagen organization through histological and two-photon microscopy analyses. Importantly, the purpose of the 3D model was not simply to confirm the biological activity observed in 2D, but to verify that the selected active concentrations remained effective after formulation, thereby reproducing the intended conditions of cosmetic application. This integrated workflow provides a stepwise human-relevant framework for translating fish-processing side-stream-derived bioactive fractions into marine-derived cosmetic active ingredients, thereby supporting their rational development from sustainable biomass to formulated cosmetic products.

2. Materials and Methods

2.1 Biomass, enzymatic hydrolysis and fractionating

Marine Hydrolyzed Peptides (MHP) were produced by NOFIMA (Tromsø, Norway) from fish processing side streams of sea bream and sea bass provided by Aqua De Mà Srl (Lavagna, Genova, Italy) as previously described (Jenssen et al. 2025). Briefly, the biomass was mixed with water and subjected to enzymatic hydrolysis in a temperature-controlled reactor using Corolase® 8000 (AB Enzymes GmbH, Darmstadt, Germany). The hydrolysis was carried out at 65°C, followed by thermal inactivation. The hydrolysate was sieved to remove bones and large particles and subsequently centrifuged to separate phases. The aqueous phase was collected, filtered to remove residual oil and fine particles. The sample was further fractionated by tangential flow filtration using a 3 kDa molecular weight cut-off membrane, yielding permeate (MHP) and retentate fractions. The MHP was freeze-dried, vacuum-packed, and stored at 4°C until further use. The molecular weight of MHP is below 3 kDa.

2.2 Chemicals

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Trizol reagent, Crystal violet (cod. 61135), chloridric acid, chloroform, and 2-propanol have been supplied by Sigma-Aldrich Chemical Company (Steinheim, Germany). Dulbecco's Modified Eagle's Medium (DMEM, high glucose w/l-glutamine w/sodium pyruvate), fetal bovine serum (FBS), L-Glutamine, trypsin, and Dulbecco's Phosphate Buffered Saline w/o calcium w/o magnesium (DPBS), used for cell culture were purchased from Euroclone (Pero, Italy). The Hyp kit (perchlorate-free) from Cell Biolabs was used for collagen quantification.

For gene expression assays, the extracted RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Biorad, Milano, Italy).

2.3 Total Protein quantification

The total protein quantification was performed using the Bradford assay. Briefly, the standard curve of Bovine Serum Albumin (BSA) was used to calculate the concentration of the protein in the MHP sample.

1 mL of Bradford reagent was mixed with 0.1 mL of tested item and read at 595 nm (Implen, Munich, Germany) after 5 min incubation in the dark at ambient temperature.

2.4 Hydroxyproline (Hyp) Quantification

Total Hyp content was determined using a perchlorate-free assay kit (Cell Biolabs, Inc., USA). Samples (100 μ L) underwent acid hydrolysis in 12 N HCl at 100°C for 3 h. After evaporation at 80°C (45 min), 10 μ L of the hydrolyzed product was reacted with 100 μ L of Chloramine T mixture (30 min, RT), followed by 100 μ L of Ehrlich's reagent (45 min, 60°C). After cooling (4°C, 5 min) and centrifugation (6000 $\times$ g, 15 min), the supernatant absorbance was measured at 560 nm (Absorbance 96, Byonoy, Germany).

2.5 Antiradical power

The antiradical power of MHP was established by the Trolox equivalent antioxidant capacity (TEAC), carrying out the ABTS assay following the protocol reported by Ferrari et al. (Ferrari et al. 2021). The antioxidant activity was expressed as micrograms of Trolox equivalents per L of extract (μ gTE/L).

2.6 Cell culture

Human Keratinocytes (HaCaT) cells were supplied by CLS Cell Lines Service GmbH (Eppelheim, Germany). Cells were cultured in Dulbecco's modified eagle's medium High Glucose (D-MEM) supplemented with 1% L-Glutamine and 10% Fetal Bovine Serum (FBS) at 37° C with 5% CO₂ in humidified air and 95% relative humidity.

The murine monocyte/macrophage cell line RAW 264.7 (ATCC TIB-71) were cultured in Dulbecco's Modified Eagle Medium (DMEM; ATCC-30-2002) supplemented with 10% heat-inactivated FBS and 1% antibiotic solution at 37°C in a 5% CO₂ atmosphere and 95% relative humidity.

2.7 Preparation of active principle S-MHP for cell treatments

The active principle MHP was initially prepared as a 10 mg/mL stock solution in distilled water (S-MHP) and subsequently diluted in culture medium to obtain different concentrations for testing on cellular models.

2.8 Formulation of S-MHP for tissues treatments

S-MHP was incorporated as an active ingredient into a cosmetic formulation (F-MHP) at final concentrations of 0.03% (w/v) and 0.1% (w/v), which was then applied to three-dimensional reconstructed human skin models in vitro. The commercial face cream employed to prepare F-MHP was kindly supplied by Ardes s.r.l. (Sarissola, Genova, Italy).

INCI: aqua (water), polyglyceryl-3 rice branate, cetearyl alcohol, glyceryl stearate, prunus amygdalus dulcis oil (prunus amygdalus dulcis (sweet almond) oil), simmondsia chinensis seed oil (simmondsia chinensis (jojoba) seed oil), glycerin, glyceryl caprylate, parfum (fragrance), hydrolyzed protein, sodium hyaluronate, rosa rugosa flower extract, sodium carboxymethyl beta-glucan, phenoxyethanol, ethylhexyl stearate, tocopheryl acetate, xanthan gum, caprylic/capric triglyceride, tetrasodium glutamate diacetate, ethylhexylglycerin, citric acid, hexamethylindanopyran, linalyl acetate, tetramethyl acetyloctahydronaphthalenes, dimethyl phenethyl acetate, geranyl acetate, benzyl alcohol, coumarin, hexyl cinnamal, hydroxycitronellal, citronellol

2.9 Cell viability assay

15×10^3 HaCaT cells were seeded in each well of a 96-well plate. After overnight incubation, cells were treated with different concentrations of MHP and incubated for 24h with 5% CO₂ at 37°C, 95% relative humidity. After that, cell viability was analyzed using MTT assay.

MTT was solubilized in PBS at a concentration of 5 mg/mL and filtered using 0.22 µm membrane. The working solution was diluted in culture medium at the final concentration of 0.5 mg/mL. The cells were then incubated for 3h in a humidified atmosphere with 5% CO₂ at 37°C. Formazan crystals were then solubilized in acid-alcohol (1 N HCl in 2-propanol) solution. The absorbance was read spectrophotometrically at 570 nm using a microplate reader (Byonoy absorbance 96, Germany).

2.10 Experimental models for curative and preventive evaluation

To comprehensively evaluate the biological efficacy of the S-MHP extract, two distinct experimental strategies were implemented, reflecting different application scenarios: The curative and preventive effect model (Fig. 1).

The curative model was designed to simulate a therapeutic intervention following established cell damage. In this experimental design, the stress stimulus (specifically for HaCaT cell: H_2O_2 1.1 mM and UVA irradiation 2.0 mW/cm², or inflammatory stimulation only for RAW 264.7 cell with LPS 100 ng/mL) was applied first to induce a state of cellular oxidative or inflammatory stress, followed by a 24h treatment with S-MHP (0.03% and 0.1% w/v). Conversely, the preventive approach was designed to assess the extract's capacity to prime cells against subsequent insults. In this model, cells (HaCaT or RAW 264.7) were pre-treated with S-MHP (0.03% or 0.1% w/v) for 24 h before being exposed to the same stress stimuli.

2.11 Evaluation of curative and preventive effects against H_2O_2 -induced oxidative stress.

To evaluate the curative and preventive effects of the active principle against oxidative stress, HaCaT cells were exposed to hydrogen peroxide (H_2O_2 , 1.1 mM) using two different experimental setups. For the curative approach, cells were first treated with H_2O_2 for 2 h, after which the oxidant compound was removed, and cells were incubated with MHP at different concentrations (0.03% and 0.1% w/v) for 24 h. For the preventive approach, cells were pre-treated with the active compound at the same concentrations for 24 h, followed by exposure to H_2O_2 (1.1 mM) for 2 h. In both cases, cell viability was subsequently assessed by MTT assay as described above.

2.12 Evaluation of curative and preventive effects against UVA-induced damage on HaCaT cells

The curative and preventive effects of the active compound against UV-induced cellular damage were investigated in HaCaT cells exposed to UVA radiation. Irradiation was performed using a solar simulator (Honle 500) equipped with an H1 filter (UVA + visible light) (OECD 2019; OECD 2026b), positioned at approximately 60 cm from the cell monolayer, providing an irradiance of 2.0–2.5 mW/cm² for 1 h. Parallel control plates were kept in the dark under the same conditions. For the curative protocol, cells were first subjected to UVA exposure and then treated with the active compound (0.03% and 0.1% w/v) for 24 h. In the preventive setting, cells were pre-incubated with the compound for 24 h prior to irradiation. Cell viability was finally determined by using the MTT assay, as previously described.

2.13 S-MHP anti-inflammatory capacity

The anti-inflammatory assay was performed using the murine monocyte/macrophage cell line RAW 264.7 (ATCC TIB-71).

Initially, cell viability was evaluated in the presence of the samples. Firstly, RAW cells were seeded at 5×10^4 cells/well in growth medium on 96-well plates and incubated for 24 h. After cells were treated with different concentrations of S-MHP, cells were incubated for 24 h, and the MTT assay was conducted as described above (supplementary, **figure S2A**).

To evaluate the immunomodulatory effects, the lipopolysaccharide (LPS) stimulation assay was performed following a previously described protocol by Jenssen et al. (Jenssen et al. 2025).

Briefly, to investigate both the preventive and curative potential of the active principle, two distinct treatment protocols were applied. For the preventive protocol, cells were pre-treated with the selected concentration of S-MHP extract before the induction of inflammation via LPS exposure (100 ng/mL). For the curative protocol, cells were first stimulated with LPS and subsequently treated with the S-MHP.

Hydrocortisone (HdC, 100 μ M) was used as positive control. Additionally, an untreated LPS-stimulated control (to monitor maximum inflammation) and a medium control (negative control) were maintained. After a 24h incubation at 37°C with 5% CO₂, cell-free supernatants were collected to measure nitric oxide (NO), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) levels. NO production was determined using a Griess reagent kit (Invitrogen Thermo Fisher, Massachusetts, United States), while IL-6 cytokine and TNF- α levels were quantified using ELISA kits (IL-6 and TNF- α ELISA Kit; Invitrogen Thermo Fisher, Massachusetts, United States) according to the manufacturer's instructions.

2.14 EpiDerm-FT culture

The normal human 3D skin model at full thickness (EpiDerm-FT; MatTek In Vitro Life Science Laboratories., Bratislava, Slovakia) comprises Normal Human Epidermal Keratinocytes (NHEK) and Normal Human Dermal Fibroblasts (NHFB), cultured to form a multilayered model of the human dermis and epidermis (Dehelean et al. 2022). Upon delivery, tissues were immediately transferred to 6-well plates and cultured in 2.5 mL DMEM (EFT-400-ASY medium) (MatTek In Vitro Life Science Laboratories). Tissues were restored in 5% CO₂ at 37°C, 95% relative humidity overnight (16–18 h). At the end of the equilibration, fresh medium was added, and EpiDerm-FT Tissues were treated topically with F-MHP (0.03% and 0.1% w/v) and then cultured at 5% CO₂, 37°C for 24 hours.

2.15 RNA Extraction

After treatments, RNA was extracted from 3D EpiDerm-FT (MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia) tissues using Trizol reagent according to Dondero et al. 2025 (Dondero et al. 2025a). Briefly, each tissue sample was homogenized in 1 mL of Trizol reagent. After allowing the mixture to incubate for 5 minutes at room temperature, 200 μ L of chloroform was added. The samples were then incubated for an additional 10 minutes at room temperature before being centrifuged at 15,294 $\times$ g for 15 minutes at 4°C, leading to the formation of three separate phases. The upper phase was carefully transferred to a new tube and mixed with 500 μ L of 2-propanol. The mixture was incubated for 10 minutes at room temperature and centrifuged again at 15,294 $\times$ g for 10 minutes at 4°C. The resulting RNA pellet was collected, washed with 1 mL of 75% ethanol, and centrifuged for 5 minutes at 5,974 $\times$ g at 4°C. After removing the ethanol, the pellet was air-dried until no residual liquid remained. Finally, the RNA pellet was dissolved in 50 μ L of RNase-free water, and both RNA concentration and purity were assessed for each sample using a Nanophotometer NP80 (IMPLEN, Munich, Germany).

2.16 cDNA synthesis and quantitative Real-Time PCR

One µg of total RNA was converted to cDNA using iScript cDNA Synthesis Kit according to the manufacturer's instructions. Expression of the following biomarkers was evaluated, based on previous studies conducted on beneficial effects of hydrolysed fish collagen Aquaporin (AQP3, commercial assay IDs: qHsaCEP0053552) (proteins involved in the transport of water, glycerol and urea across cell membrane) (McDaniel et al. 2020), Collagen Type III Alpha 1 Chain (COL3A1, commercial assay IDs: qHsaCEP0053821), Collagen Type I Alpha 1 Chain (COL1A1, commercial assay IDs: qHsaCEP0050510) (both involved in the regulation of collagen expression), Elastin (ELN, commercial assay IDs: qHsaCEP0057837) (involved in the assembly of elastic fibers) and Fibronectin-1 (FN-1, commercial assay IDs: qHsaCEP0050873) (involved in cell adhesion, migration and tissue repair) (Bio-Rad Laboratories, Hercules, CA, USA) (Katsuta 2008).

Quantitative RT-PCR was performed in triplicate using SYBR Green Supermix (Bio-Rad) and IQ supermix (Bio-Rad) on CFX96 Real-Time system (C1000 Thermal Cycler; Bio-Rad). The cDNA was subjected to PCR amplification cycle according to the following parameters: 95°C x 3min, 40 cycles at 95°C x 15s and 60°C x 40s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression served as housekeeping gene (GAPDH Fwd: 5'- GACCCCTTCATTGACCTCAAC-3'; GAPDH Rev: 5'- CGCTCCTGGAAGATGGTGTATGGG - 3').

2.17 Tissues histological analysis

EpiDerm-FT tissues subjected to the same treatments used for gene expression analysis were fixed in 10% buffered formalin (Sigma-Aldrich, Germany), rinsed in PBS (pH 7.4), dehydrated through graded ethanol solutions, cleared in Bioclear (Bio-Optica, Italy), and embedded in paraffin. Tissue sections (5 µm thick) were mounted on glass slides and stained with either hematoxylin–eosin or Sirius Red. Histological evaluation was performed using a Leica DMRB light microscope (Leica Microsystems, Wetzlar, Germany) equipped with polarized light and a Flexacam camera (Leica Microsystems, Wetzlar, Germany). Sirius Red-stained sections were used to assess collagen distribution within the dermal compartment, whereas hematoxylin–eosin-stained sections were analyzed to determine fibroblast number and dermal thickness using ImageJ software (Schneider et al. 2012).

2.18 Sirius Red staining for collagen – polarized light microscopy

Picrosirius red staining is not entirely specific for collagen, as the dye may also bind to a limited number of other tissue components. However, these structures do not exhibit birefringence under polarized light microscopy. Consequently, when sections are examined under polarized light, the method becomes highly specific for collagen fibers (Junqueira et al. 1979). Under polarized light, Sirius Red-stained sections exhibited shiny collagen fibers appearing in green, yellow, and orange against a dark background (Fig. 5).

For each treatment, five sections were imaged and the dermal region was selected using ImageJ software. Collagen content was then quantified by applying the “Color Threshold” function to generate

binary images, where white pixels corresponded to collagen fibers and black pixels to the background. The percentage of white pixels relative to the total area was used as a semiquantitative measure of collagen content.

2.19 Fibroblasts count within dermis

For each treatment, three hematoxylin–eosin-stained sections were randomly selected and imaged. In each image, three regions of interest (ROIs) of 200×200 pixels (corresponding to 0.068 mm^2 under the adopted imaging conditions) were randomly defined within the dermal area, resulting in a total of nine ROIs analyzed per treatment. Fibroblast nuclei were automatically quantified using ImageJ software. Briefly, the extracellular matrix appeared light pink to whitish, whereas fibroblast nuclei were stained purple, enabling segmentation through the “Color Threshold” function. This procedure generated binary images with a white background and black particles corresponding to fibroblast nuclei, which were subsequently counted using the “Analyze Particles” function. Objects smaller than 6 pixels were excluded to minimize artifacts related to the staining procedure. To rule out potential staining artifacts or false positives during automated particle analysis, the automated counts were manually spot-checked and verified by an experienced operator on the exact same ROIs.

2.20 Dermis thickness measurement

The thickness of the dermal portion of the tissues was randomly measured in five different hematoxylin–eosin-stained histological sections for each treatment. To avoid biases due to different cuts, we used the thickness of the filter as a normalization factor for sections that are not precisely orthogonal.

2.21 Two-photon Excitation Microscopy

EpiDerm-FT tissues treated as for the gene expression analysis were fixed in 10% buffered formalin (Sigma-Aldrich, Germany) and rinsed in PBS (pH 7.4). Two photon excitation microscopy was performed on a custom setup based on a BX51 (Olympus, Japan) upright microscope equipped with water immersion long working distance objectives (XL Plan N, $25\times$, NA = 1.05, Olympus, Japan or LUMFI $60\times$, N.A. = 1.1, Olympus, Japan). Raster scanning images were acquired through a M610 (ISS Inc., Urbana-Champaign, USA) scanning head coupled to a Ti:Sapph laser (MaiTai DeepSee, Newport, USA) and to two analog photomultiplier tubes (HC125-02, Hamamatsu, Japan). A quarter-wave plate was inserted before the objective to generate circularly polarized excitation light. Second Harmonic Generation (SHG) and Two-Photon Excitation Fluorescence (TPEF) signals were collected through bandpass filters centered at $400/40 \text{ nm}$ and $535/50 \text{ nm}$ respectively. Z-stack images were acquired from the dermal side of the tissue. As illustrated in Fig. 2A, samples were inverted during imaging on the upright microscope configuration to allow optical access from the dermal side, thereby avoiding signal attenuation and scattering from the overlying epidermal layer.

2.22 SHG imaging and quantification of the collagen fibers anisotropy

SHG signal, generated exclusively by non-centrosymmetric structures, is highly sensitive to the organization and orientation of collagen fibers (Chen et al. 2012). For all samples, label-free SHG image stacks covering a volume of $450 \times 450 \times 260 \mu\text{m}^3$ within the dermal matrix were acquired (Fig. 2A). Collagen fibers anisotropy was quantified on the SHG images by calculating the two-dimensional spatial autocorrelation function ($g(\Delta x, \Delta y)$), which measures how similar a pattern, texture, or signal is to a shifted version of itself. After subtraction of the mean image intensity,

$$\delta I_{SHG}(x, y) = I_{SHG}(x, y) - \langle I_{SHG} \rangle$$

the normalized autocorrelation function was computed according to the Wiener–Khinchin theorem:

$$g(\Delta x, \Delta y) = \frac{F^{-1} \left\{ |F[\delta I_{SHG}(x, y)]|^2 \right\}}{\langle \delta I_{SHG}^2 \rangle}$$

where F and F^{-1} denote the two-dimensional Fourier transform and inverse Fourier transform, respectively. A representative autocorrelation map is shown in Fig. 2B. The elongated shape of the central autocorrelation peak indicates a preferential alignment of collagen fibers. The peak was fitted with a two-dimensional asymmetric Lorentzian function (Fig. 2C-E):

$$L(x, y) = \frac{a}{1 + \frac{x^2}{d_m^2} + \frac{y^2}{d_M^2}} + background$$

where d_M and d_m are the characteristic widths of the fitted peak along the major and minor principal axes, respectively. Collagen anisotropy was quantified as the ratio $A = d_M/d_m$ where $A = 1$ corresponds to an isotropic collagen distribution, and larger values indicate increasing preferential alignment of collagen fibers. All image processing and quantitative analyses were performed using custom Python scripts.

2.23 TPEF imaging and quantification of the nuclei area fraction

Fibroblast nuclei were stained with Hoechst to perform TPEF imaging covering a volume of $150 \times 150 \times 260 \mu\text{m}^3$ within the dermal matrix. Samples were first permeabilized with 0.1% Triton X-100 (Mattek, Slovakia) in water for 1 h. Nuclei were then stained with Hoechst 33342 (ThermoFisher Scientific, Italy) in a dilution ratio of 1:1000 in PBS for 40 min. After staining, samples were thoroughly washed in PBS for 1 h to remove excess dye prior to imaging. Two-photon excited fluorescence (TPEF) imaging of cell nuclei was subsequently performed as described above. Nuclear area fraction was quantified from TPEF images using intensity-based segmentation. Specifically, binary masks were obtained using Otsu's thresholding method implemented in the `skimage.filters.threshold_otsu` Python routine. The nuclear area fraction was then calculated as the ratio between the total segmented nuclear area and the total image area.

2.24 Statistical analysis

Statistical analysis was performed using GraphPad Prism 10 (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were compared using one-way ANOVA using Tukey’s post hoc testing. A p value < 0.05 was considered statistically significant. Results are presented as mean ± SD.

3. Results

3.1 Preliminary Characterization of S-MHP

The first phase of this study involved the biochemical characterization of the S-MHP. Quantitative analysis revealed a protein concentration of $29.36 \pm 6.34 \mu\text{g/mL}$. A significant portion of this protein content was identified as collagen-derived, as evidenced by a hydroxyproline concentration of $12.5 \pm 2.12 \mu\text{g/mL}$. Hydroxyproline represented approximately 42% of the measured protein concentration, indicating a substantial collagen-associated component (Shahidi and Saeid 2025; Jeyachandran and Aman 2025; Gallo et al. 2026). Additionally, the antioxidant capacity of the extract was evaluated via TEAC assay, revealing a Trolox equivalent concentration of $1.253 \pm 0.002 \mu\text{g/L}$ (Table 1.). This baseline antioxidant activity provides a preliminary chemical rationale for the cytoprotective effects against oxidative stress (H2O2) and UV radiation subsequently observed in the cellular models.

Table 1
Protein, hydroxyproline content and Antioxidant property of S-MHP.

Sample	Protein Concentration [$\mu\text{g/mL}$]	Hydroxyproline Concentration [$\mu\text{g/mL}$]	Trolox equivalent Concentration [$\mu\text{g/L}$]
S-MHP	29.36 ± 6.34	12.5 ± 2.12	1.253 ± 0.002

3.2 Cytoprotective and Restorative Effects of S-MHP against H2O2 and UV-Induced Stress in HaCaT Cells

To evaluate the biocompatibility and bioactivity of S-MHP, HaCaT cells were initially exposed to a wide range of concentrations (0.006–0.25% w/v). The MTT assay revealed no cytotoxic effects across the tested range (Fig. 3A). Notably, concentrations of 0.03%, 0.05%, and 0.1% w/v induced a statistically significant increase in cell viability compared to the negative control (CTRL), suggesting enhanced cellular viability under basal conditions of the fish-derived peptides on human keratinocytes. Based on the results of the cell viability assay, the 0.03% and 0.1% (w/v) concentrations were selected for subsequent experiments.

To further investigate whether the proliferative effects observed under basal conditions translated into protection against skin-relevant insults, the cytoprotective potential of S-MHP was evaluated using both oxidative and UV-induced stress models.

In the H₂O₂ curative model (Fig. 3B), exposure to 1.1 mM H₂O₂ for 2 h induced a marked and statistically significant reduction in HaCaT cell viability compared with the negative control (CTRL), confirming the establishment of oxidative damage. Treatment with S-MHP for 24 h after the oxidative insult significantly restored cell viability at both 0.03% and 0.1% (w/v) compared with the H₂O₂-treated positive control. Notably, the 0.1% concentration not only restored cell viability but increased it to values significantly higher than those observed in the negative control, suggesting that S-MHP exerted biological effects extending beyond the recovery of H₂O₂-induced damage. In the H₂O₂ preventive model (Fig. 3D), pre-treatment with S-MHP for 24 h significantly protected HaCaT cells against the subsequent oxidative challenge. Both concentrations effectively prevented the H₂O₂-induced loss of viability, restoring cell viability to levels comparable with the negative control.

A similar response was observed when HaCaT cells were subjected to UVA-induced stress (2.0 mW/cm² for 1 h). Exposure to UVA alone induced a significant reduction in cell viability compared with the negative control (CTRL), confirming the establishment of photo-induced cellular damage. In the curative protocol (Fig. 3C), treatment with S-MHP at both 0.03% and 0.1% (w/v) after UVA exposure significantly restored cell viability at both tested concentrations compared with the UV-treated positive control. The 0.1% concentration produced the greatest recovery, restoring cell viability to values significantly higher than the negative control. In the preventive protocol (Fig. 3E), pre-treatment with S-MHP significantly protected HaCaT cells against the subsequent UVA challenge. Both concentrations significantly improved cell viability compared with the UV-treated positive control. Nevertheless, cell viability remained significantly lower than that of the negative control, indicating that S-MHP attenuated, but did not completely prevent, UVA-induced cytotoxicity.

To verify that the observed effects, after UVA exposure, were not influenced by light-induced modifications of the active ingredient during irradiation, the photostability of S-MHP was evaluated in a cell-free system and compared with two reference solar organic filters, Benzophenone-3 (Bp3) and Benzophenone-4 (Bp4) (Supplementary **Figure S1**). S-MHP was exposed to the same irradiation conditions used in the cellular assays for 60 min in the absence of cells, and its absorbance profile was subsequently analyzed. Spectrophotometric analysis at 270 nm for S-MHP and 290 nm for Bp3 and Bp4 showed no relevant changes in S-MHP absorbance after exposure to the solar simulator, suggesting that the irradiation protocol did not induce detectable light-dependent modifications of the active ingredient as compared with Bp3 and Bp4. These findings support the stability of S-MHP under the experimental UV stress conditions used in this study.

3.3 Anti-inflammatory capacity of S-MHP on RAW 264.7 cells

To investigate the immunomodulatory potential of the S-MHP, RAW 264.7 murine macrophages were used as an in vitro inflammatory model to evaluate its effects on LPS-stimulated macrophages in terms of nitrite, IL-6 and TNF- α production under both curative and preventive conditions (Fig. 4).

In the curative protocol, stimulation with LPS alone induced a marked and statistically significant increase in nitrite production, IL-6 secretion and TNF- α release compared with the negative control, confirming the establishment of an inflammatory response. In the LPS curative model (Fig. 4A–C), when S-MHP was administered after LPS stimulation, its anti-inflammatory efficacy was limited. Regarding nitrite production, only the highest concentration (0.1% w/v) induced a slight but significant reduction compared with the LPS-treated positive control ($p < 0.05$; Fig. 4A). In contrast, post-treatment with S-MHP did not reduce the production of the pro-inflammatory cytokines IL-6 and TNF- α (Fig. 4B, 4C). Surprisingly, S-MHP post-treatment was associated with a significant increase in IL-6 secretion at 0.1% ($p < 0.0001$) and TNF- α secretion at 0.03% ($p < 0.01$) compared with the LPS-treated positive control. Moreover, for all inflammatory parameters evaluated in the curative protocol, values remained significantly higher than those of the negative control, indicating that the inflammatory response was not fully reversed by post-treatment with S-MHP.

In contrast, in the preventive protocol, pre-treatment with S-MHP demonstrated an anti-inflammatory effect. Exposure of RAW 264.7 cells to LPS alone induced an increase in nitrite production, as well as IL-6 and TNF- α secretion, compared to the negative control. Pre-treatment with S-MHP significantly attenuated all these inflammatory parameters. Specifically, the 0.1% concentration significantly reduced nitrite production ($p < 0.0001$; Fig. 4D). Likewise, S-MHP significantly decreased IL-6 and TNF- α secretion. For IL-6, the 0.03% concentration exerted a greater inhibitory effect than the 0.1% concentration (Fig. 4E), whereas both concentrations significantly reduced TNF- α release ($p < 0.01$; Fig. 4F). Despite these protective effects, all S-MHP-treated groups remained significantly different from the negative control, indicating that pre-treatment attenuated, but did not completely prevent, the inflammatory response induced by LPS. These findings demonstrate that S-MHP effectively mitigates, although does not fully abolish, macrophage inflammatory activation under the experimental conditions tested.

3.4 Modulation of Collagen Synthesis in a 3D Skin Model

To evaluate the efficacy of the formulated product, F-MHP was topically applied to a reconstructed full-thickness human skin model (RHE), comprising both epidermis and dermis compartments. The corresponding placebo formulation, consisting of the same cosmetic base without added MHP, was used as the negative control, allowing the specific contribution of the active ingredient to be distinguished from that of the formulation itself. Gene expression analysis performed on RHE treated with F-MHP at 0.03% and 0.1% (w/v) revealed a significant modulation of the two major fibrillar collagen genes. Specifically, COL3A1 mRNA expression showed a dose-dependent increase, reaching statistical significance at both tested concentrations (Fig. 5A). Interestingly, COL1A1 expression was markedly upregulated at the lower concentration (0.03% w/v), while returning to negative control levels at 0.1% w/v (Fig. 5B), indicating a differential modulation of COL1A1 and COL3A1 expression by F-MHP. The observed modulation of collagen-related genes was paralleled by increased collagen deposition, as evidenced by Sirius Red staining (Fig. 5C). Qualitative evaluation under polarized light and subsequent quantitative image analysis (Fig. 5D) confirmed a significant increase in total collagen content following treatment with 0.1% (w/v) F-MHP.

These findings were independently supported by label-free second harmonic generation (SHG) microscopy. Unlike conventional histological staining, SHG provides a label-free and highly specific readout of fibrillar collagen, enabling the simultaneous evaluation of collagen abundance and spatial organization within the reconstructed dermal matrix (Chen et al. 2012). Representative three-dimensional SHG reconstructions (Fig. 5F) revealed a denser and more aligned collagen network in F-MHP-treated samples compared to the untreated control. Quantitative analysis of the integrated SHG signal confirmed a significant increase in fibrillar collagen content at the 0.1% w/v dose (Fig. 5G), consistent with the histological quantification obtained by Sirius Red staining (Fig. 5D). Together, these results indicate that the transcriptional upregulation observed at the gene expression level translated into increased collagen accumulation and maturation within the complex tissue environment.

Beyond collagen abundance, SHG imaging was used to quantify collagen fiber anisotropy, a parameter describing the degree of preferential fiber alignment and structural organization of the extracellular matrix (see Materials and Methods). The organization of collagen fibers is a major determinant of dermal tissue quality, as the architecture of the collagen network contributes to the mechanical strength and functional integrity of healthy skin (Cicchi et al. 2010). As shown in Fig. 5E, treatment with F-MHP at both tested concentrations significantly increased collagen anisotropy compared with the control condition. The higher anisotropy values indicate the formation of a more ordered and aligned collagen network, suggesting that F-MHP not only stimulates collagen synthesis but also promotes the assembly of a structurally organized extracellular matrix. Collectively, these findings demonstrate that the addition of MHP to the cosmetic formulation enhances both the quantity and architectural quality of collagen within the dermal compartment.

3.5 Fibroblast proliferation on dermal portion of 3D skin model

To assess the effect of the F-MHP treatment on the cellular component of the dermal compartment, fibroblast abundance was evaluated using two complementary approaches, namely conventional histology and multiphoton fluorescence imaging (Fig. 6). Histological examination of H&E-stained sections from the reconstructed 3D skin models (Fig. 6A) revealed an increase in fibroblast density following F-MHP treatment. Quantitative analysis showed that fibroblast density was significantly increased only in tissues treated with 0.1% (w/v) F-MHP compared with the negative control, whereas the 0.03% (w/v) concentration did not differ significantly from the control (Fig. 6B). These findings suggest that the higher F-MHP concentration may support dermal cellularity under the experimental conditions used. To independently assess fibroblast abundance within the dermal compartment, fibroblast nuclei were stained with Hoechst 33342 and imaged by two-photon excitation fluorescence microscopy. Representative three-dimensional reconstructions of the dermal region up to 260 μm depth are shown in Fig. 6C. Quantification of the nuclei area fraction (Fig. 6D) revealed a significant increase in the fraction of tissue occupied by fibroblast nuclei following treatment with F-MHP, with the highest effect observed at the 0.1% w/v dose. This result is consistent with the increase in fibroblast density

measured by H&E staining, providing independent confirmation of the enhanced cellularity of the dermal compartment.

3.6 Enhancement of Dermal Architecture, Elasticity, and Hydration Markers on 3D skin model

To further characterize the regenerative effects of the formulated product (F-MHP) on RHE, the expression of key genes involved in skin elasticity and hydration was investigated. The results revealed a significant upregulation of Fibronectin-1 (FN1), Elastin (ELN), and Aquaporin-3 (AQP3) mRNA expression exclusively at the lower concentration tested. Specifically, tissues treated with 0.03% (w/v) F-MHP showed a statistically significant increase in FN1 (Fig. 7A), ELN (Fig. 7B), and AQP3 (Fig. 7C) expression compared with the control. These findings suggest an enhancement of the adhesive glycoprotein network and elastic component of the extracellular matrix, together with the activation of molecular pathways associated with skin hydration and barrier function (De Luca et al. 2016). Notably, these transcriptional effects were observed only at the lower F-MHP concentration.

On the contrary, histological analysis (Fig. 7D) revealed a significant increase in dermal thickness only in tissues treated with 0.1% (w/v) F-MHP compared with the control (Fig. 7E). This effect was accompanied by a significant increase in fibroblast density at the same concentration (Han et al. 2025).

4. Discussion and conclusion

Beyond the biological characterization of a single marine hydrolysate, the present work proposes a stepwise human-relevant strategy for the development and efficacy assessment of marine-derived cosmetic active ingredients obtained through circular valorization. The proposed workflow (Fig. 8) integrates biomass valorization, biochemical characterization, active-ingredient screening in simplified cellular models, formulation development, and tissue-level efficacy assessment in reconstructed human skin models. Rather than representing independent experimental steps, these approaches provide complementary information, progressively supporting the translation of marine side streams into candidate cosmetic active ingredients. Within this framework, the present study demonstrates the feasibility of transforming a low-molecular-weight protein hydrolysate obtained from sea bream and sea bass side streams into a marine-derived cosmetic active exhibiting measurable biological efficacy in both simplified cellular models and advanced reconstructed human skin models.

The workflow integrates biomass valorization, extraction and fractionation, biochemical characterization of the active fraction, efficacy screening of the active ingredient in 2D cellular models relevant to skin protection and inflammation (HaCaT keratinocytes and RAW 264.7 macrophages), formulation development and efficacy assessment of the formulated product in reconstructed 3D human skin models. The stepwise approach enables the translation of marine side streams into candidate cosmetic active ingredients through a scientifically robust, human-relevant efficacy assessment strategy.

Following the proposed workflow, the first level of evaluation focused on the biochemical characterization of the active fraction, providing the biochemical basis for its subsequent biological assessment. The biochemical characterization of S-MHP confirmed its nature as a protein-rich, low-molecular-weight marine fraction with a relevant collagen-associated component, as indicated by its hydroxyproline content. Hydroxyproline is a distinctive marker of collagen-rich matrices (Eastoe 1957); however, S-MHP should not be interpreted as an exclusively collagen-derived material, but rather as a complex mixture of protein-derived peptides in which collagen-associated sequences coexist with other bioactive peptides. This compositional complexity may contribute to the multifunctional biological responses observed in the subsequent human-relevant models.

The second step of the workflow consisted of identifying biologically active and non-cytotoxic concentrations of marine hydrolysate. HaCaT keratinocytes were initially exposed to a broad concentration range of S-MHP. Among the tested concentrations, 0.03% and 0.1% (w/v), corresponding to the lowest and highest concentrations capable of significantly increasing basal cell viability, were selected for subsequent investigations. This preliminary screening provided the rationale for selecting biologically active concentrations to be further evaluated under skin-relevant stress conditions. The selected concentrations were subsequently investigated in simplified 2D cellular models to determine whether the increase in basal cell viability was translated into biologically relevant protective responses. In HaCaT keratinocytes, S-MHP was evaluated as an aqueous solution against oxidative and photo-induced stress. Both concentrations significantly protected cells from H₂O₂-induced oxidative stress and UVA-induced damage, demonstrating that the active ingredient retains cytoprotective activity under conditions relevant to skin ageing (Vibhute et al. 2023). Interestingly, the lower concentration was already sufficient to exert protective effects comparable to those observed at the higher dose, suggesting that the biological activity of S-MHP is not directly proportional to concentration in simplified cellular models.

The second component of the 2D evaluation focused on the immunomodulatory properties of S-MHP. RAW 264.7 macrophages stimulated with LPS were employed to compare the preventive and curative activity of the active ingredient. A clear difference emerged between the two experimental approaches. When administered after the inflammatory stimulus, S-MHP showed limited anti-inflammatory activity, producing a modest reduction in nitrite production at the highest concentration while failing to reduce IL-6 and TNF- α secretion. Moreover, post-treatment increased IL-6 or TNF- α release at selected concentrations. Conversely, pre-treatment with S-MHP significantly attenuated the subsequent inflammatory response, reducing nitrite production, IL-6, and TNF- α secretion. The contrasting outcomes of the preventive and curative protocols indicate that the immunomodulatory activity of S-MHP is strongly dependent on macrophage activation state and on the timing of peptide exposure relative to the inflammatory stimulus. Interestingly, a remarkably similar context-dependent response has been reported for collagen-derived peptides in RAW 264.7 macrophages: peptide pre-incubation before LPS challenge tended to attenuate inflammation, whereas administration after LPS activation enhanced IL-6 and TNF- α production and NF- κ B signaling (Li et al. 2022). Moreover, different collagen hydrolysates can produce distinct cytokine profiles in macrophages, indicating that peptide composition may further

influence the direction of the immune response (Fernandes et al. 2025). Thus, S-MHP appears primarily effective in modulating the establishment of the inflammatory response, whereas administration after LPS activation may interact with an already established inflammatory program and enhance selected cytokine responses. The mechanisms underlying this context-dependent effect remain to be determined. Importantly, these findings should not be interpreted as evidence of a general pro-inflammatory effect of S-MHP, since the direction of the response differed markedly according to the sequence of exposure. Rather, they highlight the importance of experimental context when assessing the immunomodulatory activity of complex peptide mixtures. Together with the cytoprotective data obtained in keratinocytes, these results demonstrate that the 2D models not only allowed the identification of biologically effective concentrations but also provided information on the biological profile of the active ingredient before formulation.

The information generated during the 2D evaluation was subsequently translated into formulation development. The concentrations identified as biologically active were incorporated into a cosmetic emulsion (F-MHP) and evaluated in a reconstructed full-thickness human skin model. This transition did not simply represent an increase in biological complexity, but also a shift from evaluating the active ingredient in solution to assessing the efficacy of the final formulated product under conditions that more closely resemble topical cosmetic application.

Gene expression analysis demonstrated that the lower concentration of F-MHP induced the broadest transcriptional response, significantly modulating extracellular matrix-related genes involved in collagen organization, elasticity, and hydration (Cole et al. 2018; Jiang et al. 2025). In contrast, histological analyses revealed that the higher concentration produced more evident structural changes, including increased fibroblast density and dermal thickness, while both concentrations improved collagen fiber organization, as demonstrated by increased anisotropy measured by two-photon microscopy. These findings indicate that the two concentrations do not simply differ in efficacy but rather generate distinct biological response profiles. Whereas the lower concentration preferentially modulated molecular pathways associated with extracellular matrix homeostasis, the higher concentration appeared to exert more pronounced effects on tissue architecture.

A particularly relevant observation emerging from this study is therefore the absence of a simple linear concentration-response relationship. Non-linear concentration-response profiles have also been reported for collagen-derived peptides. In human dermal fibroblasts, Dierckx et al. observed greater COL1A1 and VCAN induction at 0.01% than at 1% collagen peptides after 24 h; notably, collagen accumulation was also markedly greater at the lower concentration, indicating that increasing peptide concentrations do not necessarily result in proportionally greater biological responses (Dierckx et al. 2024). Several non-mutually exclusive mechanisms could contribute to this behavior, including concentration-dependent feedback regulation or saturation of peptide-responsive cellular pathways. In the present 3D model, additional complexity may arise from the incorporation of MHP into a cosmetic emulsion and its topical application, as concentration-dependent differences in peptide release, diffusion through the reconstructed tissue and local bioavailability may influence the responses of different cellular and tissue

compartments. These mechanisms were not directly investigated and therefore remain hypotheses requiring dedicated mechanistic studies.

The apparent discrepancy between transcriptional and structural responses should also be considered in relation to their different biological kinetics. In particular, COL1A1 was significantly upregulated at 0.03% but not at 0.1% F-MHP, whereas COL3A1 increased at both concentrations. Although 24 h represents a relatively short exposure period, early changes in collagen metabolism can occur within this time frame. Collagen-derived peptides have been reported to induce collagen-related gene expression and measurable collagen synthesis/secretion in dermal fibroblasts within 8–24 h (Tokudome et al. 2012; Dierckx et al. 2024), and we previously observed increased COL1A1/COL3A1 expression together with enhanced collagen deposition after a single 24-h treatment in the same reconstructed full-thickness skin model (Dondero et al. 2025a). Importantly, mRNA expression measured at a single 24-h endpoint does not necessarily parallel extracellular collagen accumulation at the same time point, since collagen abundance reflects the integrated outcome of transcription, synthesis, secretion/deposition and turnover over the preceding period. Accordingly, the increased fibrillar collagen detected at 0.1% F-MHP cannot be attributed specifically to COL3A1 upregulation, nor does the absence of significant COL1A1 upregulation at 24 h exclude a contribution of type I collagen.

The interpretation of collagen birefringence under polarized light remains a matter of debate, as different colors have been associated with collagen maturity, packing density or fibril thickness in different studies. Because no consensus has been reached regarding the biological significance of these color differences, all birefringent fibers were considered as collagen irrespective of their color (Piérard 1989). This conservative approach allowed us to focus on the overall abundance of organized collagen, minimizing interpretative bias while maintaining a robust comparison among treatments (Lattouf et al. 2014).

Interestingly, while Sirius Red staining provides information on collagen abundance, SHG-derived anisotropy reflects the spatial organization of the collagen network. Therefore, the increase in anisotropy observed following F-MHP treatment suggests that collagen remodeling involves not only greater matrix deposition but also the establishment of a more organized fibrillar architecture. Such an organized collagen network has been associated with improved mechanical competence of connective tissues, although dedicated biomechanical studies would be required to directly demonstrate this effect. The combination of Sirius Red staining and SHG imaging therefore provide complementary information on both the quantity and the quality of collagen remodeling induced by F-MHP.

Dermal thickness should be interpreted with appropriate caution. Reconstructed full-thickness skin models inherently exhibit a certain degree of variability in dermal thickness between individual tissues due to the manufacturing process. Consequently, this parameter is generally considered less robust than molecular or biochemical endpoints. Nevertheless, despite these intrinsic limitations, the increase in dermal thickness observed at 0.1% (w/v) F-MHP was in agreement with the independent histological and imaging data, including the increase in fibroblast density, collagen deposition and collagen fiber

organization. For this reason, dermal thickness was not interpreted as an isolated endpoint but rather as part of an integrated evaluation including molecular, histological, and biophysical analyses.

In conclusion, this study demonstrates that sea bream and sea bass by-products can be converted into a low-molecular-weight marine protein hydrolysate with measurable biological activity and concrete cosmetic application potential. Moreover, the distinctive attribute of the S-MHP extract investigated in this study lies in its capacity to exert remarkable biological efficacy across multiple protective and immunomodulatory pathways already upon a first topical application, highlighting its strong potential as a fast-acting active ingredient for cosmetic formulations. Indeed, S-MHP was biocompatible and cytoprotective in human keratinocytes exposed to oxidative and UVA-induced stress, highlighting its potential for anti-pollution cosmetic formulations and as a complementary photoprotective active or sunscreen booster. Finally, once incorporated into a cosmetic formulation, it modulated extracellular matrix-, elasticity- and hydration-related markers in a reconstructed 3D human skin model. Moreover, the observed effects on dermal architecture, collagen deposition, fibroblast number and collagen fiber organization support its relevance as a functional cosmetic active compound.

More broadly, this study demonstrates that marine side streams can move beyond the concept of waste and be transformed into scientifically validated, high-value cosmetic ingredients through a human-relevant biological validation strategy. Beyond the specific marine hydrolysate investigated, the integrated workflow presented here provides a transferable framework for the biological validation of marine-derived cosmetic ingredients obtained from circular bioeconomy processes. Future studies should investigate whether incorporation of MHP into sunscreen formulations could reduce the amount of conventional UV filters required while maintaining photoprotective efficacy, thereby contributing to a One Health approach that integrates human skin protection with environmental sustainability.

Declarations

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Figures

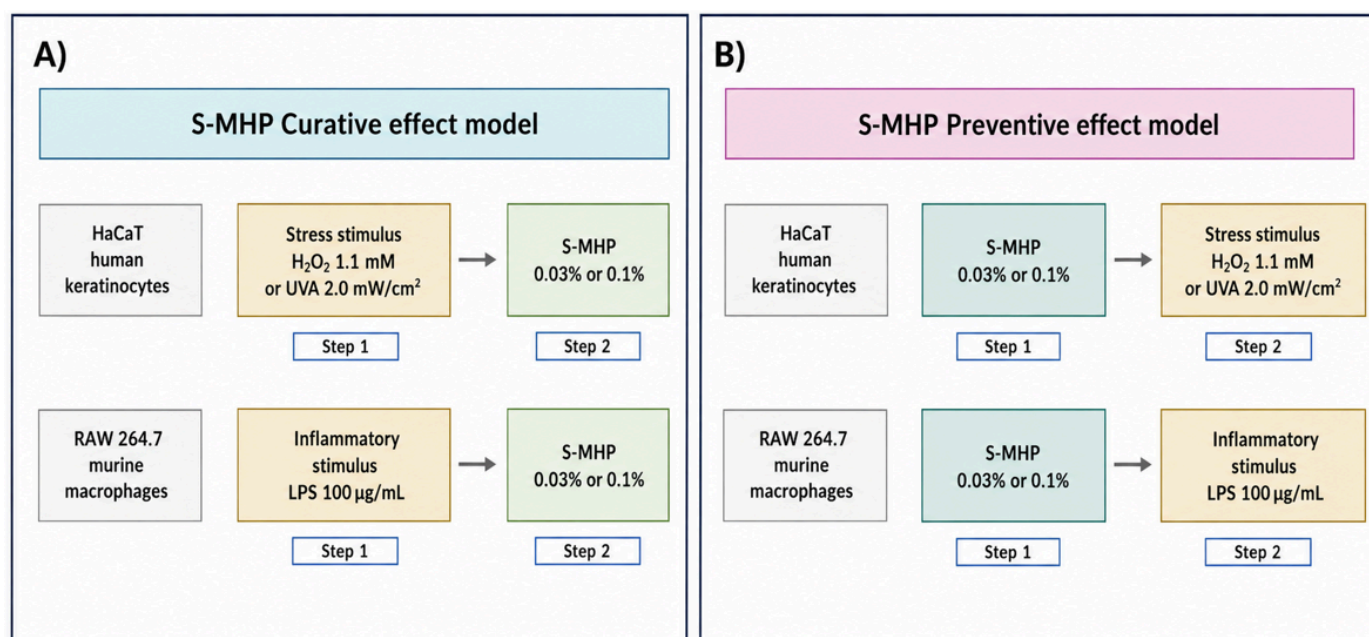


Figure 1

Schematic representation of the experimental design for assessing the biological activity of S-MHP. (A) Curative model, where the stress stimulus was applied initially to induce cellular damage, followed by a 24 h post-treatment with S-MHP (0.03% or 0.1% w/v). (B) Preventive effect model, where cells were pre-treated with S-MHP (0.03% or 0.1% w/v) for 24 h prior to the application of the stress stimulus. Both models were applied to HaCaT and RAW 264.7 cells to evaluate the versatility and efficacy of the marine-derived active principle in both curative and protective contexts.

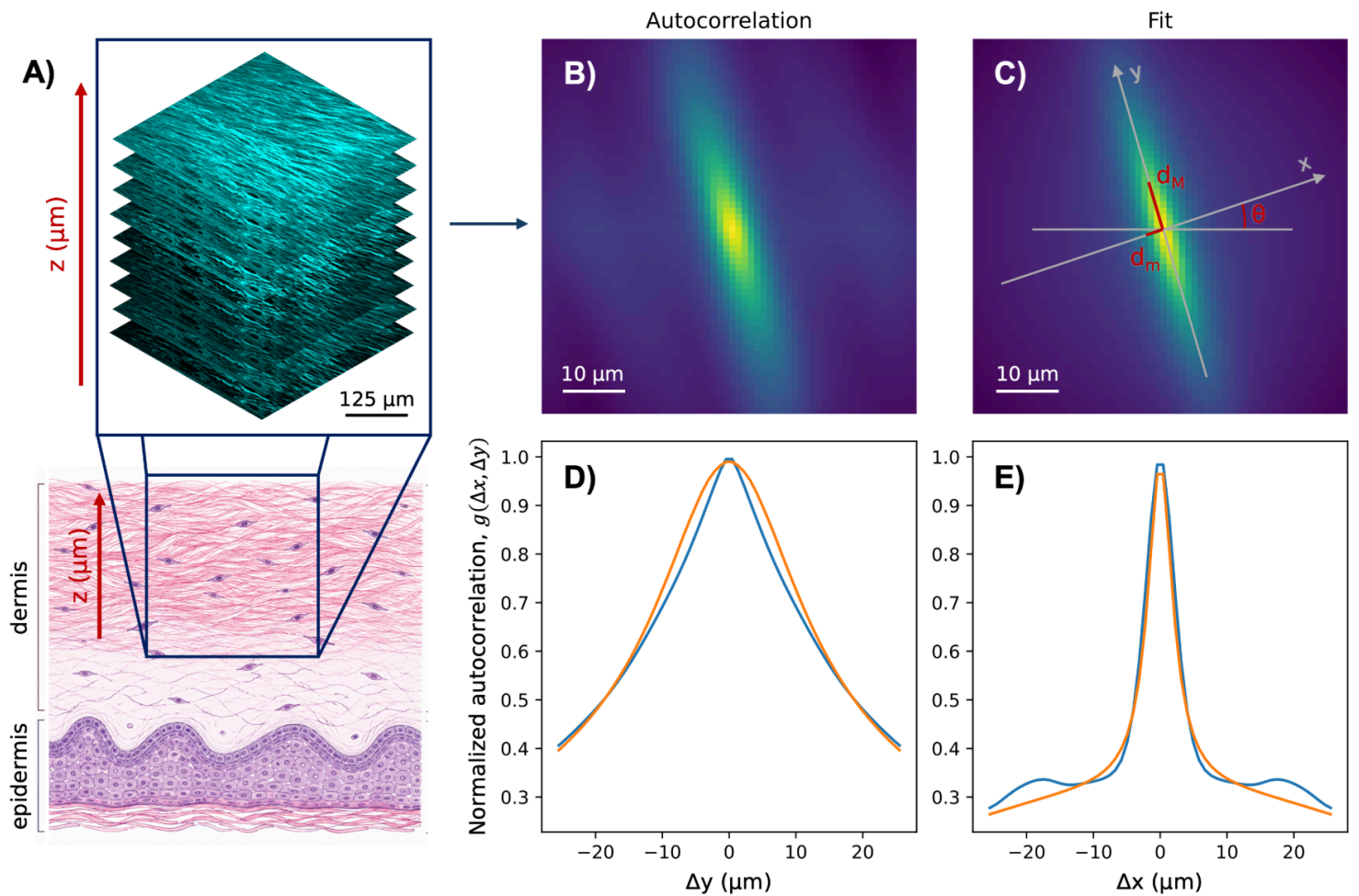


Figure 2

Schematic workflow for SHG image acquisition and quantitative analysis of collagen fiber anisotropy. (A) Representative SHGg(Δx , Δy), showing an elongated central peak indicative of preferential collagen fiber alignment. (C) Two-dimensional asymmetric Lorentzian fit of the autocorrelation peak. The fitted surface is shown together with its principal axes, rotated by an angle θ with respect to the image reference frame. The major and minor principal directions are indicated in gray, while the corresponding characteristic widths d_m are shown in red. (D–E) Cross-sections of the autocorrelation peak (blue) together with the Lorentzian fit (orange) along the major and minor principal axes, respectively.

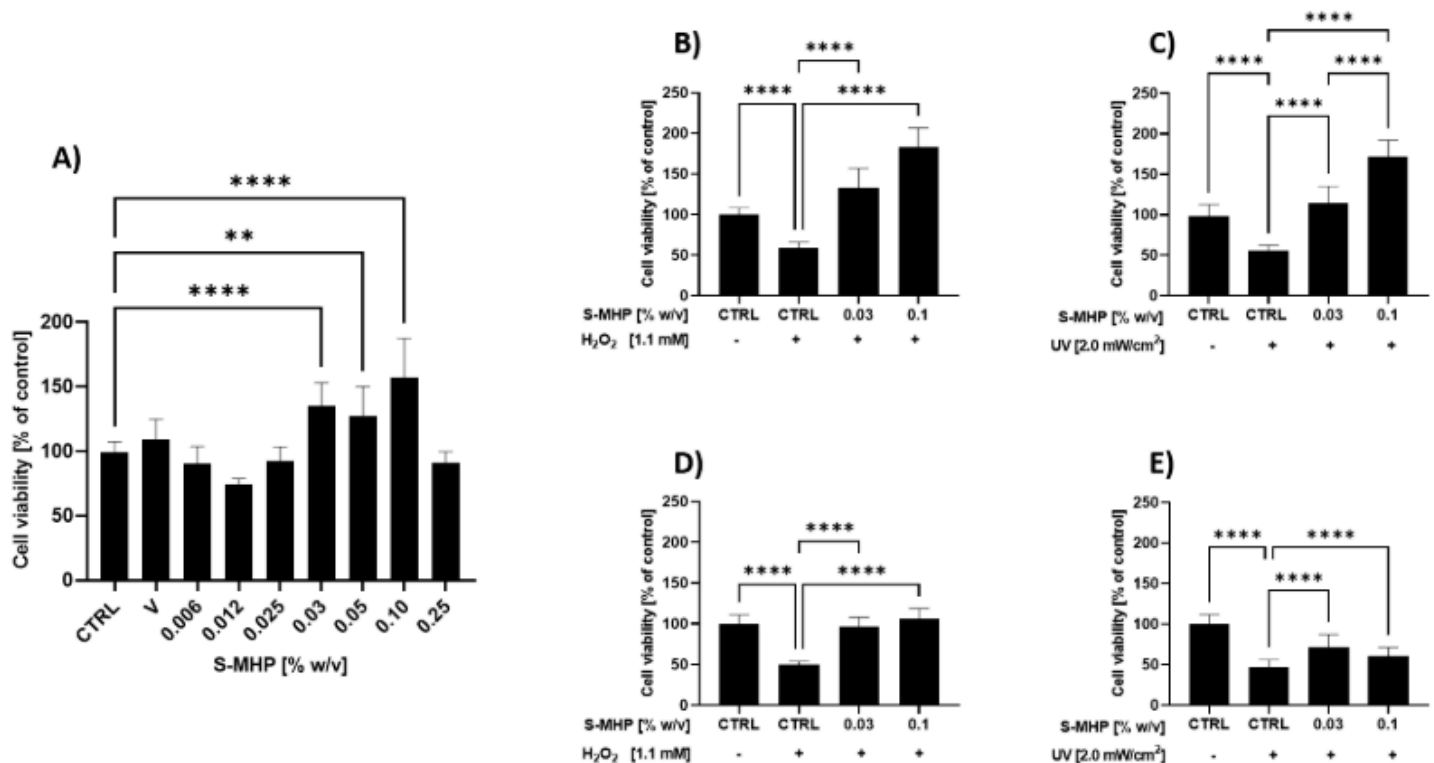


Figure 3

Evaluation of cell viability and cytoprotective effects of the S-MHP sample on HaCaT cells. (A) MTT assay of HaCaT cells treated 24h with different concentrations of S-MHP (0.006–0.25% w/v). (B) Curative effect against oxidative stress: cells were exposed for 2h to H₂O₂, followed by 24 h of treatment with S-MHP (0.03% and 0.1% w/v). (C) Curative effect against UV stress: cells were irradiated with UVA (2.0 mW/cm²) for 1 h, followed by 24 h of treatment with S-MHP. (D) Preventive effect against oxidative stress: cells were pre-treated with the S-MHP for 24 h before 2 h of H₂O₂ exposure. (E) Preventive effect against UV stress: cells were pre-treated with S-MHP for 24 h before UVA irradiation (2.0 mW/cm²). Cell viability was assessed by MTT assay and expressed as a percentage (%) relative to the negative control (CTRL). Data are presented as mean $\pm$ SD of at least three independent experiments. The significant differences are indicated by symbols on bars (** $p \leq 0.0001$, * $p \leq 0.01$ vs CTRL). Other significant differences not indicated in the figure are reported below. Figure 3A: **$p \leq 0.0001$** : V vs 0.10%, 0.006% vs 0.10%, 0.012 vs 0.03, 0.012 vs 0.05, 0.012 vs 0.10, 0.025 vs 0.10, 0.10 vs 0.25; **$p \leq 0.001$** : 0.006 vs 0.03, 0.025 vs 0.03, 0.05 vs 0.10; **$p \leq 0.01$** : V vs 0.03, 0.03 vs 0.10, 0.03 vs 0.25; **$p \leq 0.05$** : V vs 0.012, 0.006 vs 0.05; 0.025 vs 0.05. Figure 3B: **$p \leq 0.0001$** : CTRL- vs 0.03%; CTRL- vs 0.1%; 0.03% vs 0.1%; Figure 3C: **$p \leq 0.0001$** : CTRL- vs 0.03%; CTRL- vs 0.1%; 0.03% vs 0.1%; Figure 3E: **$p \leq 0.0001$** : CTRL- vs 0.03%; CTRL- vs 0.1%; 0.03% vs 0.1%; Cells were treated with S-MHP (0.03% or 0.1% w/v). CTRL- represents untreated cells (vehicle only), whereas CTRL+ represents cells exposed to the stress stimulus (H₂O₂ or UVA) without S-MHP treatment.

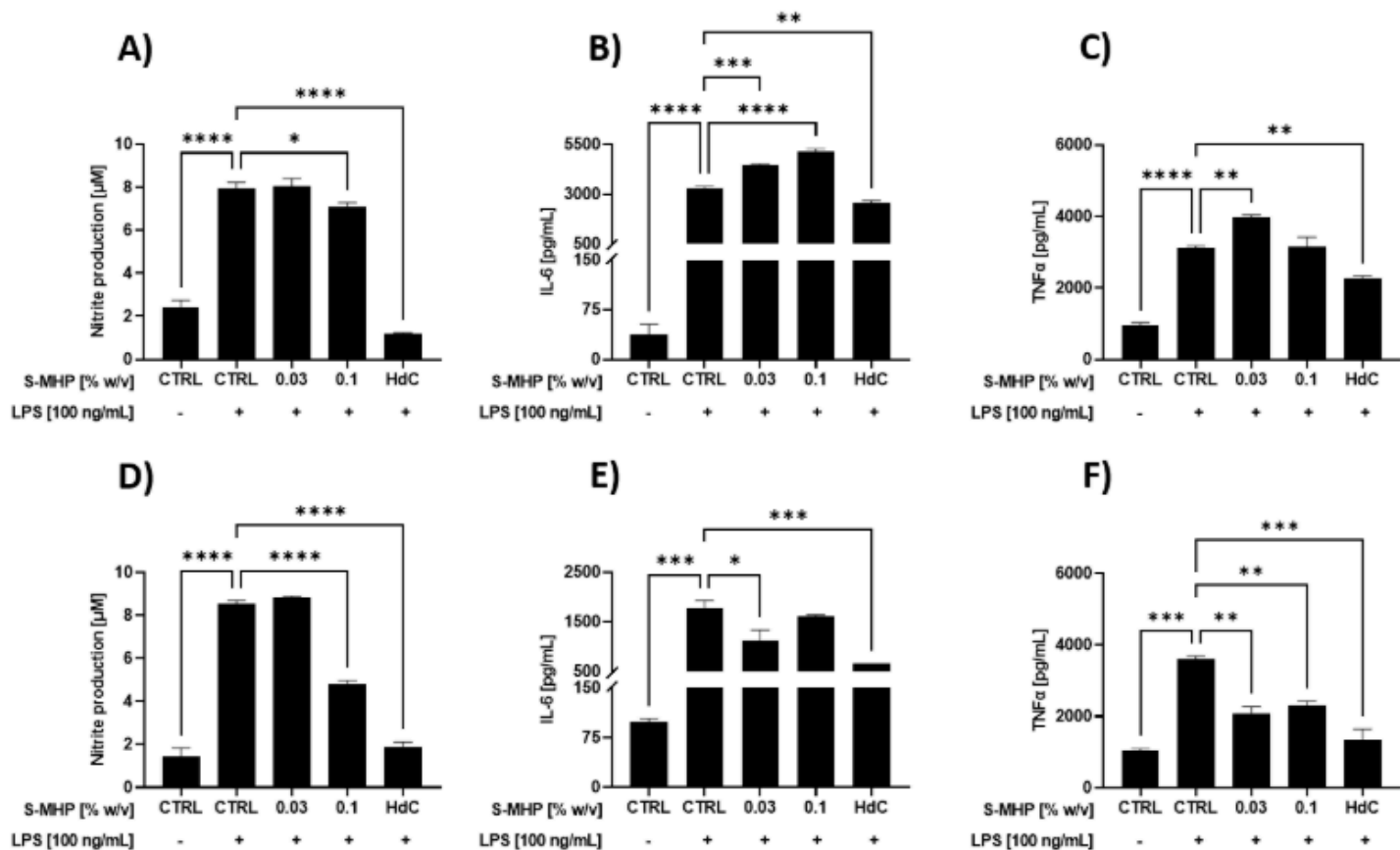


Figure 4

Evaluation of Anti-inflammatory capacity of S-MHP on RAW 264.7 cells under LPS-induced inflammation (100 ng/mL). The restorative potential of the active principle was assessed by exposing cells to LPS followed by 24h treatment with S-MHP (A, B, C). The preventive capacity was evaluated by pre-treating cells with S-MHP for 24h prior to LPS stimulation (D, E, F). The following inflammatory markers were quantified: Nitrite production (μM) via Griess assay (A and D); Interleukin-6 (IL-6) levels (pg/mL) via ELISA assay (B and E); Tumor Necrosis Factor-α (TNF- α) levels (pg/mL) via ELISA assay (C and F). Hydrocortisone (HdC) was employed as a positive anti-inflammatory control. Data are presented as mean ± SD of at least three independent experiments. The significant differences are indicated by symbols on bars (**** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$ vs CTRL+). Other significant differences not indicated in the figure are reported below. Figure 4A: **$p \leq 0.0001$** : CTRL- vs 0.03%; CTRL- vs 0.1%; CTRL- vs HdC; 0.03% vs HdC; 0.1% vs HdC; **$p \leq 0.01$** : 0.03% vs 0.1%; Figure 4B: **$p \leq 0.0001$** : CTRL- vs 0.03%; CTRL- vs 0.1%; 0.03% vs HdC; 0.1% vs HdC; **$p \leq 0.001$** : CTRL- vs HdC; **$p \leq 0.01$** : 0.03% vs 0.1%; Figure 4C: **$p \leq 0.0001$** : CTRL- vs 0.03%; CTRL- vs 0.1%; **$p \leq 0.001$** : CTRL- vs HdC; 0.03% vs HdC; **$p \leq 0.01$** : 0.03% vs 0.1%; 0.1% vs HdC; Figure 4D: **$p \leq 0.0001$** : CTRL- vs 0.03%; CTRL- vs 0.1%; 0.03% vs 0.1%; 0.03% vs HdC; **$p \leq 0.01$** : CTRL- vs HdC; 0.1% vs HdC; Figure 4E: **$p \leq 0.001$** : CTRL- vs 0.1%; **$p \leq 0.01$** : CTRL- vs 0.03%; 0.1% vs HdC; **$p \leq 0.05$** : CTRL- vs HdC; 0.03% vs 0.1%; 0.03% vs HdC; Figure 4F: **$p \leq 0.01$** : CTRL- vs 0.03%; CTRL- vs 0.1%; **$p \leq 0.05$** : 0.03% vs HdC; 0.1% vs HdC; Cells were treated with S-MHP (0.03% or 0.1% w/v). CTRL- represents untreated cells (vehicle only), whereas CTRL+ represents cells exposed to the stress stimulus (LPS) without S-MHP treatment.

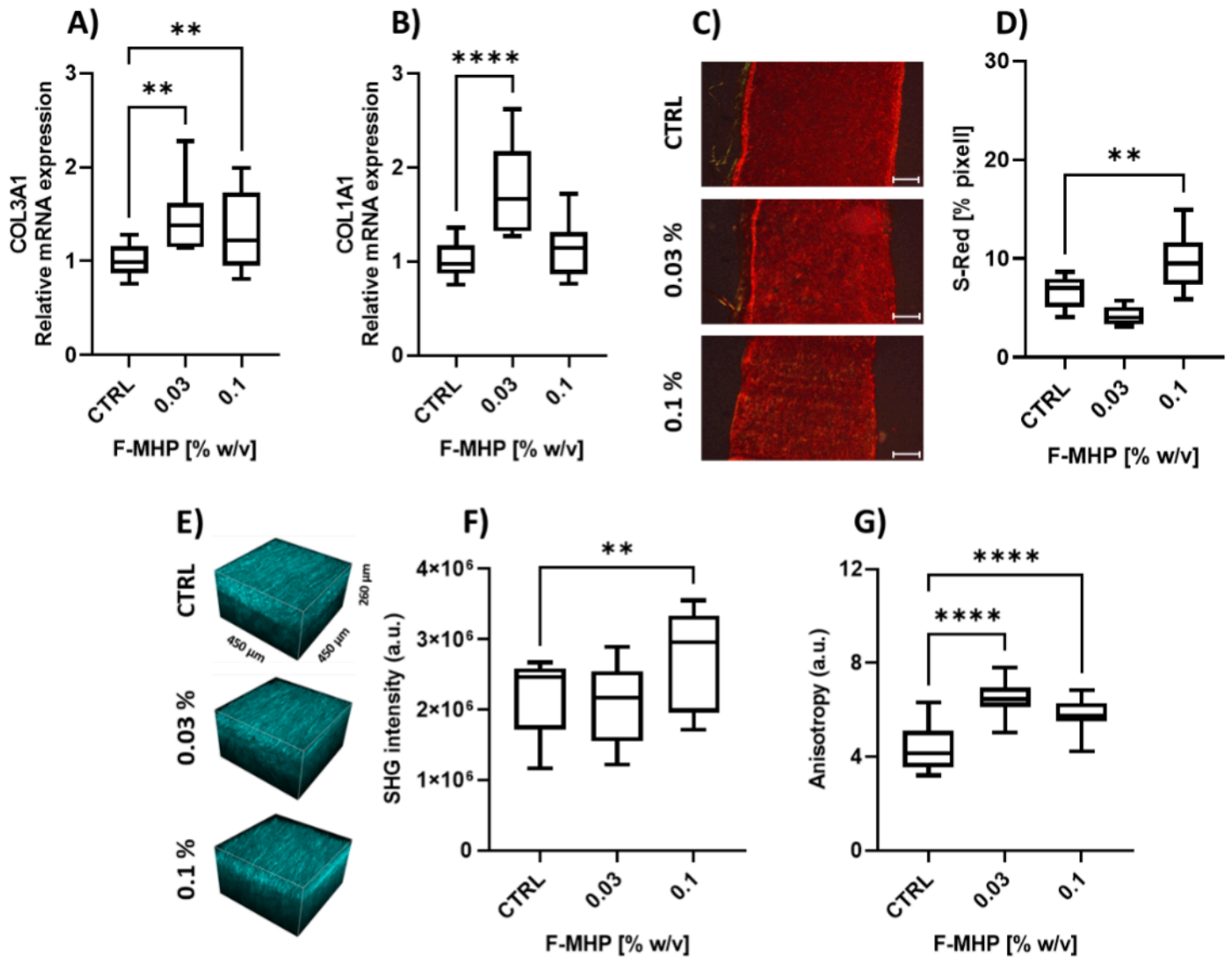


Figure 5

Effect of F-MHP on collagen expression and tissue architecture in a 3D skin model. (A) Relative mRNA expression of COL3A1 and (B) COL1A1 after 24 h of treatment, normalized to the control. (C) Representative images of Sirius Red (S-Red) staining for the visualization of collagen fibers; Scale bar: 124 μ m. (D) Quantification of S-Red signal intensity expressed as a percentage (%) of pixels per field. (E) Representative volumetric reconstructions of Second Harmonic Generation (SHG) signal acquired by two-photon microscopy showing the structural organization of collagen fibers in the dermal matrix. (F) Quantification of the integrated SHG intensity in the dermal layers from two-photon microscopy images. (G) Quantification of collagen fiber anisotropy derived from SHG images. Box plots represent the median, interquartile range, and min-max values of at least three independent replicates. The significant differences are indicated by symbols on bars (**** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$ vs CTRL). Other significant differences not indicated in the figure are reported below. Fig. 5B: **$p \leq 0.0001$** : 0.03% vs 0.1%; Fig. 5D: **$p \leq 0.0001$** : 0.03% vs 0.1%; Fig. 5G: **$p \leq 0.001$** : 0.03% vs 0.1%; Samples were treated with F-MHP

(0.03% or 0.1% w/v). CTRL corresponds to the placebo formulation, consisting of the same cosmetic base without MHP.

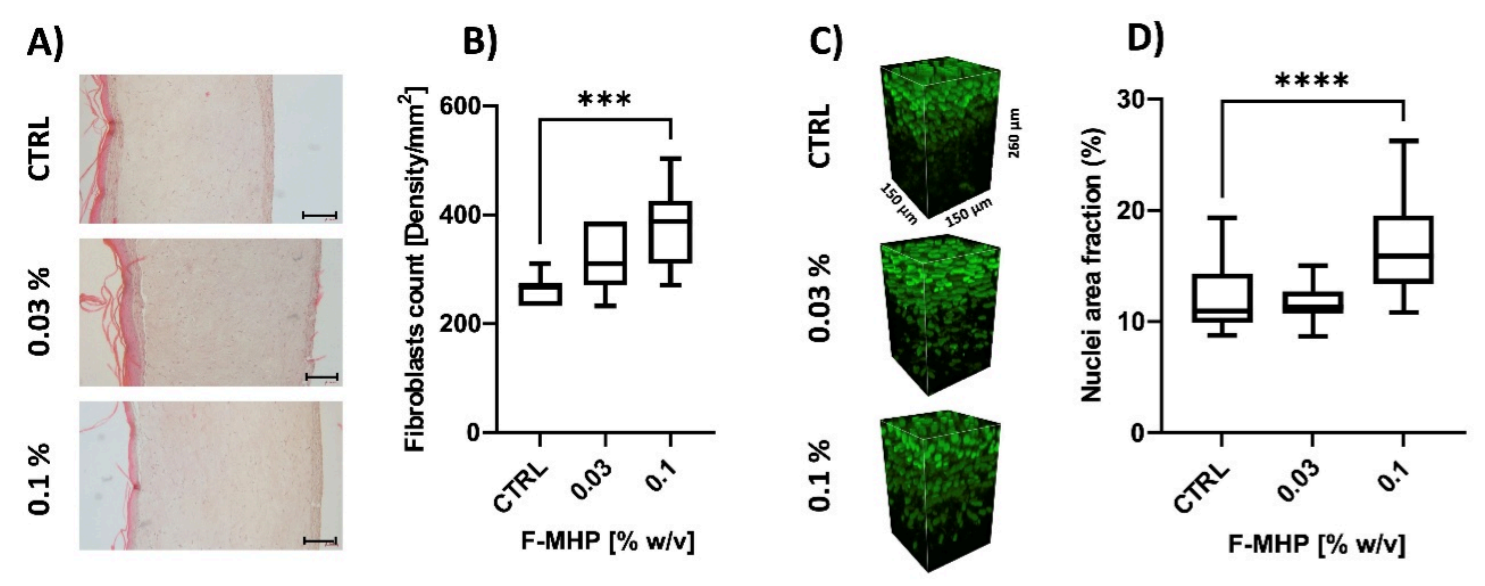


Figure 6

Effect of F-MHP on Fibroblasts count and Nuclei area fraction in a 3D skin model. (A) Representative H&E images of Fibroblasts count (density/mm²) in a 3d skin models; Scale bar: 124 μ m. (B) Quantitative measurement of Fibroblasts count (density/mm²) across different experimental groups. (C) Representative volumetric reconstructions of Hoechst-stained fibroblast nuclei acquired by two-photon fluorescence microscopy. (D) Quantification of the nuclei area fraction in the dermal compartment from two-photon fluorescence microscopy images. The significant differences are indicated by symbols on bars (**** p $\leq$ 0.0001, *** p $\leq$ 0.001 vs CTRL). Other significant differences not indicated in the figure are reported below. Fig. 6D: **p $\leq$ 0.0001**: 0.03% vs 0.1%; Samples were treated with F-MHP (0.03% or 0.1% w/v). CTRL corresponds to the placebo formulation, consisting of the same cosmetic base without MHP.

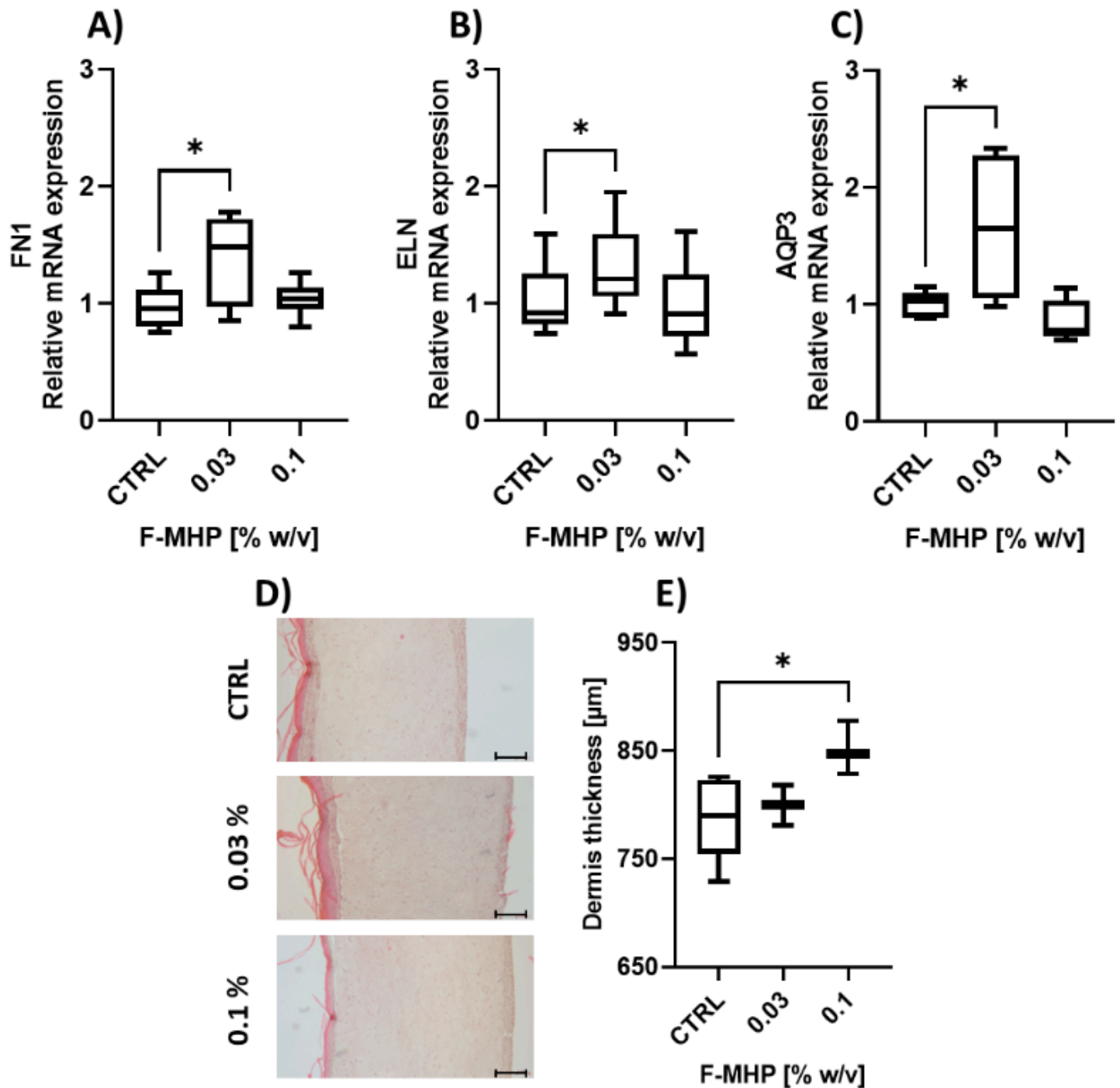


Figure 7

Impact of F-MHP on dermal ECM components, hydration markers, and dermis thickness. (A) Relative mRNA expression of FN1 (Fibronectin 1), (B) ELN (Elastin), and (C) AQP3 (Aquaporin-3) in the 3D skin model after 24 h of treatment. (D) Representative H&E-stained histological sections highlighting the dermal compartment; Scale bar: 124 μm . (E) Quantitative measurement of dermis thickness (μm) across the different experimental groups. The significant differences are indicated by symbols on bars (** $p \leq 0.01$, * $p \leq 0.05$ vs CTRL). Other significant differences not indicated in the figure are reported below. Fig. 7A: **$p \leq 0.05$** : 0.03% vs 0.1%; Fig. 7C: **$p \leq 0.01$** : 0.03% vs 0.1%; Samples were treated with F-

MHP (0.03% or 0.1% w/v). CTRL corresponds to the placebo formulation, consisting of the same cosmetic base without MHP.

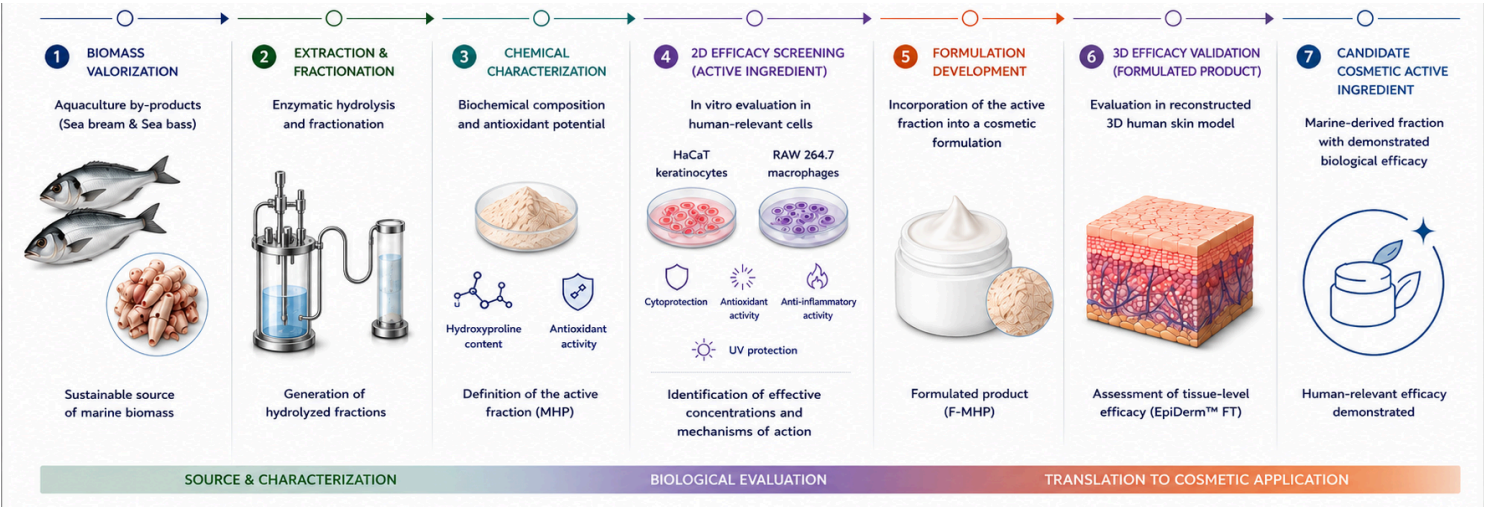


Figure 8

Proposed stepwise workflow for the development and efficacy assessment of marine-derived cosmetic active ingredients.

Supplementary Files

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