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"Promoting Skin Longevity and Wellbeing using a Cannabisin-rich Eco-extract from Bioreactor-cultivated Hemp"

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1. Introduction

In a context where the pursuit of longevity and well-being has become a societal priority, developing innovative natural active ingredients is essential to meet the growing demand for improved quality of life. Moreover, the increasing demand for natural and sustainable skincare solutions is leading us to explore the potential of hemp-derived compounds for promoting skin longevity and general well-being. Cannabisin F, a bioactive compound found mainly in hemp (*Cannabis sativa)* seeds and fruits, is known for its antioxidant, anti-inflammatory, and neuroprotective properties [1]. A novel approach to hemp hairy roots production in a controlled bioreactor system has demonstrated a hundred-fold increase in cannabisin F production, using 74 times less space and 91 times less water when compared to hemp seeds [2].

This study explores the efficacy of a cannabisin-rich extract derived from bioreactor-cultivated *Cannabis sativa* hairy roots (CSHR) in stimulating key biological pathways associated with skin cells' longevity, including the maintenance of proteostasis, stimulation of autophagy, and protection against senescence [3]. First, a microwave-assisted extraction was performed on the CSHR biomass, and its performance was compared with an extraction without the use of microwaves. Then, the obtained dried extract was applied to human skin explants, and its efficacy was assessed through the evaluation of:

- Protein carbonylation levels in skin sections, associated with oxidative damage in each skin compartment [4];
- LAMP2A levels, related to chaperone-mediated autophagy, detoxification, and tissue repair [5];
- Sirtuin-1 levels, linked to detoxification processes and nutrient sensing [6];
- Number of $P16^{INK4A}$ positive cells, a biomarker associated with senescence [7];
- Keratin-14 levels, a biomarker associated with epidermal integrity and renewal [8];
- TNF-α and IL-6 levels, two pro-inflammatory cytokines associated with the inflammatory response and even with chronic inflammation [9].

2. Materials and Methods

2.1. Hairy root biomass cultivation and extraction process

The process of cultivating and preparing root biomass begins with the co-cultivation of *Cannabis sativa* plantlets with *Agrobacterium rhizogenes* wild-type (WT) strains. After co-cultivation, root clones are isolated. These are individual root segments that will be used for further cultivation. The isolated root clones are then inoculated into a sterile, disposable bag containing a growth medium. The growth medium includes essential nutrients such as water, sugar, and vitamins to support the growth of the root biomass. The inoculated root clones are then cultivated in a bioreactor for four weeks. After the cultivation period, the root biomass is harvested and subjected to vacuum freezing at -20°C before being sent for the extraction process. The cultivation and preparation process are depicted in Figure 1.

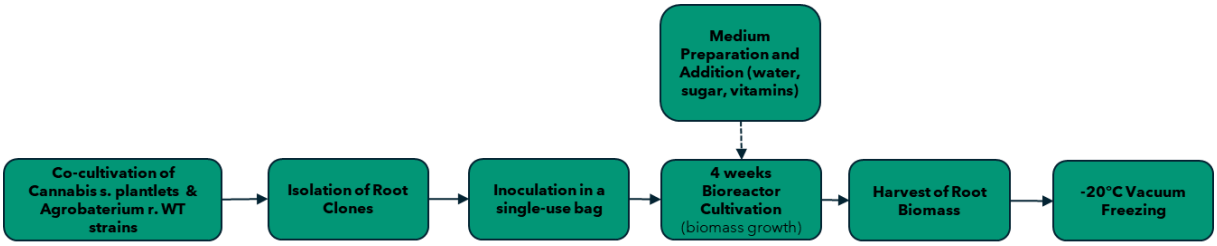

Figure 1: Cultivation procedure to obtain *Cannabis sativa* hairy roots (CSHR).

The dry CSHR extract was made using ethanol and water (50/50 in weight) as solvents and compared with an extraction process using microwaves. Microwave extraction was carried out in a vessel coupled to a 75-kW microwave generator emitting microwaves at a frequency between 2,450 GHz and 0.915 GHz. Microwaves and stirring are activated for 30 minutes. A solid/liquid separation is carried out to recover the liquid extract. Finally, successive filtrations

are carried out on stainless steel mesh to achieve a filtration porosity of 25 µm, followed by freeze-drying, as shown in Figure 2.

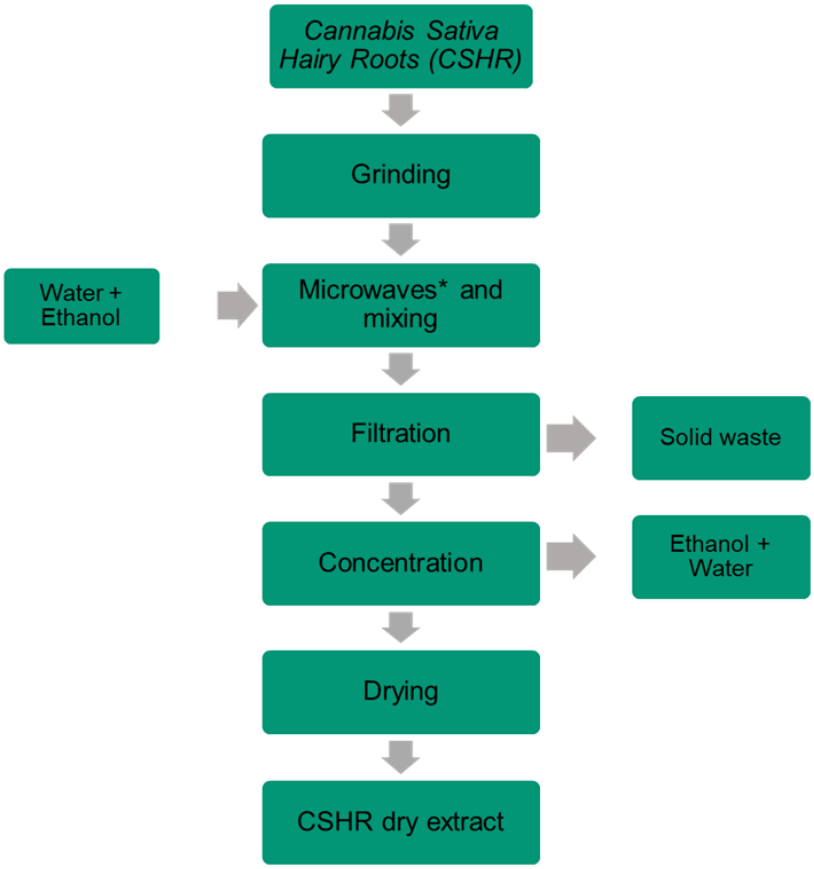

Figure 2: Extraction procedure to obtain *Cannabis sativa* hairy root (CSHR) dry extract.

*Microwave-assisted extraction was compared to a classical extraction without microwaves.

The CSHR was analyzed using liquid chromatography (UHPLC Elute, Bruker) followed by mass spectrometry (HRMS High-resolution mass spectrometer, TIMS-ToF). First, an HSS T3 column Waters at 35°C with a Mobile phase: gradient ultrapure water – methanol, Injection volume: 1 µL was used. For the detection, a 10 mg sample of the dried extract was dissolved in 10 mL of methanol and put in an ultrasonic bath for 15 minutes with shaking and then filtered at 0.2 µm PTFE.

2.2. Ex vivo efficacy tests

Skin explants were obtained with informed consent from the abdominal surgery of a 31-year-old female Caucasian donor (phototype II/III). After surgery, they were kept alive by culturing in OxiProteomics® medium at 37 °C in 5% CO2 humidified air. The skin explants were distributed in 9 experimental groups (n=3 *per* group; Table 1), and the culturing medium was renewed every 24 hours.

Table 1. Experimental groups for the ex vivo tests

Description	Treatment(s)	Sampling
Control	Not treated No stress	24h after the treatment
0.003% CSHR extract 0.001% CSHR extract 1% Resveratrol	Topically treated with products (30 µL/cm2) for 24h No Stress	
Stress (UVA)	Not treated UV-A irradiation (λ=365 nm; 6 J/cm²)	3h and 24h after the stress
0.003% CSHR extract + Stress 0.001% CSHR extract + Stress 1% Resveratrol + Stress	Topically treated with products (30µL/cm2) for 24h UV-A irradiation (λ=365 nm; 6 J/cm²)	

Explant sections of 5 µm in thickness were obtained using a cryostat (Leica). Skin sections were fixed with a solution containing 95% Ethanol and 5% acetic acid and then *in situ* protein carbonylation was labeled by using a fluorescent probe (Ex = 647 nm / Em = 650 nm) functionalized to specifically bind to carbonyl moieties [4]. DAPI (4',6-diamidino-2-phenylindole) was employed for nuclear labeling.

For immunodetection, skin sections were fixed with a solution containing 4% paraformaldehyde, followed by a permeabilization step using 0.1% Triton for 10 min. A saturating step of the non-specific sites was carried out with a solution of BSA (Bovine Serum Albumin) at 3% in PBS (Phosphate Buffered Saline, pH 7.4). Tissue sections were incubated with a primary antibody for each biomarker detection in a 3% BSA solution in PBS (Table 3). The excess of primary antibody was eliminated with washing steps with 3% BSA solution in PBS, then the explant sections were incubated for 1 hour with the secondary antibody coupled to a fluorophore. The cellular nuclei were labelled with DAPI. Finally, the antibody and DAPI excess were removed with a sequence of washing steps with PBS.

A series of fluorescent images was collected with an epi-fluorescent microscope (EVOS M5000 Imaging System; 40x objective) using strictly the same acquisition time and resolution per series. The raw source images were collected, including the complete range of fluorescence signal intensity (.TIFF 16-bit format), then analyzed using ImageJ software (Schneider, 2012). The intensity of targeted biomarker levels was obtained by the integration of the specific fluorescence signal over threshold, normalized by the evaluated area. The intensity of carbonylation levels was obtained by the integration of the specific fluorescence

signal obtained for the different anatomical compartments (dermis, epidermis, and stratum corneum) and for the overall skin. For $P16^{INK4A}$ positive cells evaluation, the quantification was carried out on each image by the integration of the number of positive labeled cells normalized by the length of the evaluated skin sections. The quantification of Sirtuin-1 levels was carried out using nuclei as the region of interest (ROI). Accurate measurement of protein concentration was carried out using the Bradford Protein Assay Dye Reagent (Biorad™) accordingly to the manufacturer's guidelines. Extracted proteins were distributed in equal amounts *per* sample and then separated by high-resolution electrophoresis (SDS-PAGE - gradient 4-20%; Thermo Scientific™). After migration, proteins were transferred from the gel to a 0.2 µm Nitrocellulose membrane (BioRad™ 1620233). To visualize the correct transfer of loaded proteins, the membrane was incubated with ponceau red solution (Thermo Scientific™) for 5- 10 min before being washed several times with MilliQ water. The membrane was washed in a Tris-buffered saline (TBS) solution at pH 7.6 with 0.1% of tween (TBS-T) before being incubated for 30 min in TBS with 3% of Bovine Serum Albumin (TBS-BSA) for a saturation step.

The quantification of biomarkers was normalized in relation to the control (considered at 100%), to finally obtain a mean value and a standard deviation. Statistical analyses were carried out using the "GraphPad" software (La Jolla, California, USA) by using One-way ANOVA and Dunnett's post-hoc test for multiple-comparison analyses or using an Unpaired t test with Welch's correction vs Control or Stress group (confidence interval of 95%). An efficacy value (%) was obtained for the experimental groups using as references the control group considered at maximum efficiency (100%), and the stress (UV-A) group at minimum efficiency (0%).

$$\textbf{Efficacy}\ \%\ (\text{group } X) = \frac{\text{Biomarker level (Stress)} - \text{Biomarker level (group X)}}{\text{Biomarker level (Stress)} - \text{Biomarker level (Control)}} * 100$$

An induction value (%) was obtained for the experimental groups using the following formula:

$$\textbf{Induction}\ \%\ (\text{group } X) = \frac{\text{Biomarker level (group X)}}{\text{Biomarker level (Control)}} - 1 * 100$$

3. Results and Discussion

3.1. Hairy root biomass cultivation and extraction process

Using the microwaves, the dried CSHR extract contained a 46 times higher concentration of cannabisin F, when compared to a CSHR extract obtained without the microwaves, as shown in Table 2. Considering firstly the 100-fold increase in cannabisins due to HR technology, this

implies that the combination of the two allows recovery of almost 4600 times more cannabisin F in a CSHR microwave-assisted extract, when compared to a regular hemp seed extract.

Table 2. Cannabisin F concentration for the two extraction methods.

CSHR Sample	Concentration (*µg / g*)
With microwaves	112
Without microwaves	3

3.2. Ex vivo efficacy tests

3.2.1. Protein carbonylation

In-situ detection of carbonylation level (visualized in red) was performed by epifluorescence microscopy (Figure 3) on the condition treated at 0.001% with the CSHR extract. The stress (UVA) increased the levels of oxidized protein. The presence of the CSHR extract counteracted the stress-mediated increase in carbonylation levels.

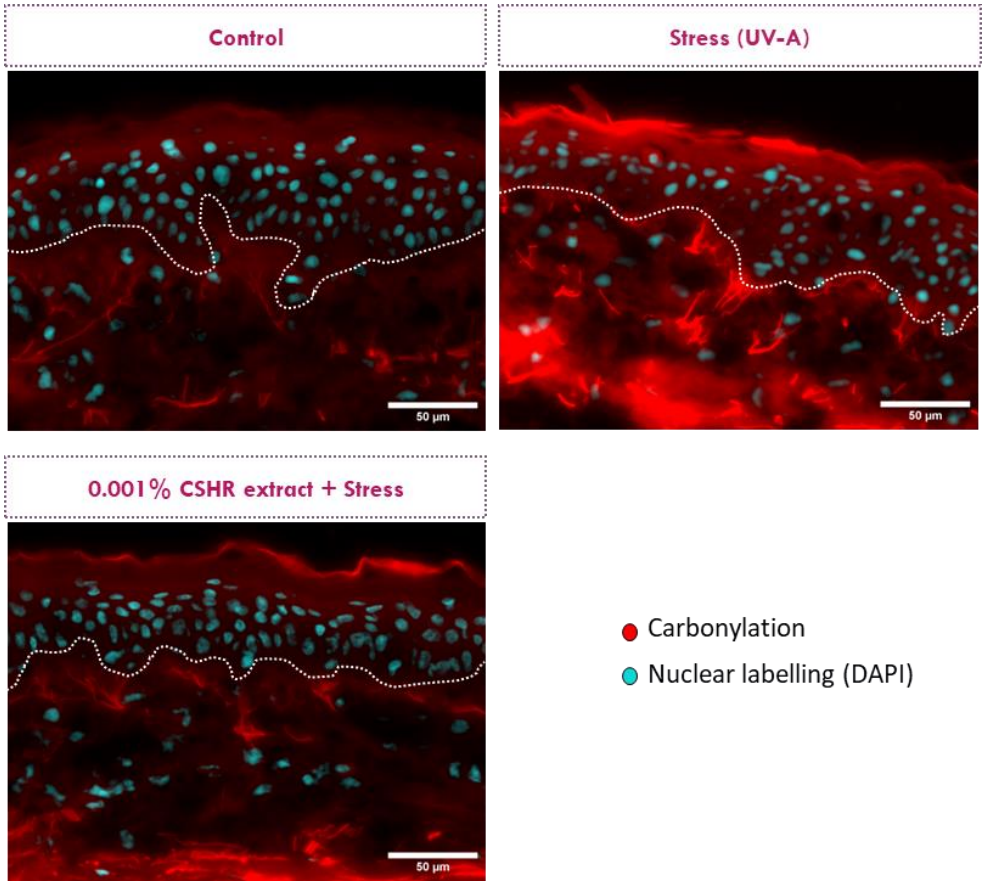

Figure 3: *In-situ* visualization of Carbonylation levels. The white dotted line separates the epidermis and the dermis.

Compared to the control, stress (UV-A) significantly increased the carbonylation levels. The presence of the CSHR extract significantly protects against oxidative stress induced by UV-A irradiation, resulting in proteostasis improvement, as revealed by 85% efficacy at 0.001%. The analysis by compartments gives 93% efficacy in the stratum corneum, 100% efficacy in the epidermis, and 78% efficacy in the dermis.

3.2.2. LAMP2A levels

Compared to the control group, the presence of CSHR extract significantly increased LAMP2 levels (+47% induction), suggesting a stimulation of autophagy and the detoxification process, which could contribute to improved skin longevity.

3.2.3. Sirtuin-1 levels

In-situ detection of sirtuin-1 levels (visualized in yellow) was performed by epifluorescence microscopy (Figure 4). The stress (UVA) decreased the levels of sirtuin-1. The presence of the CSHR extract and resveratrol counteracted the stress-mediated decrease in the levels of sirtuin-1.

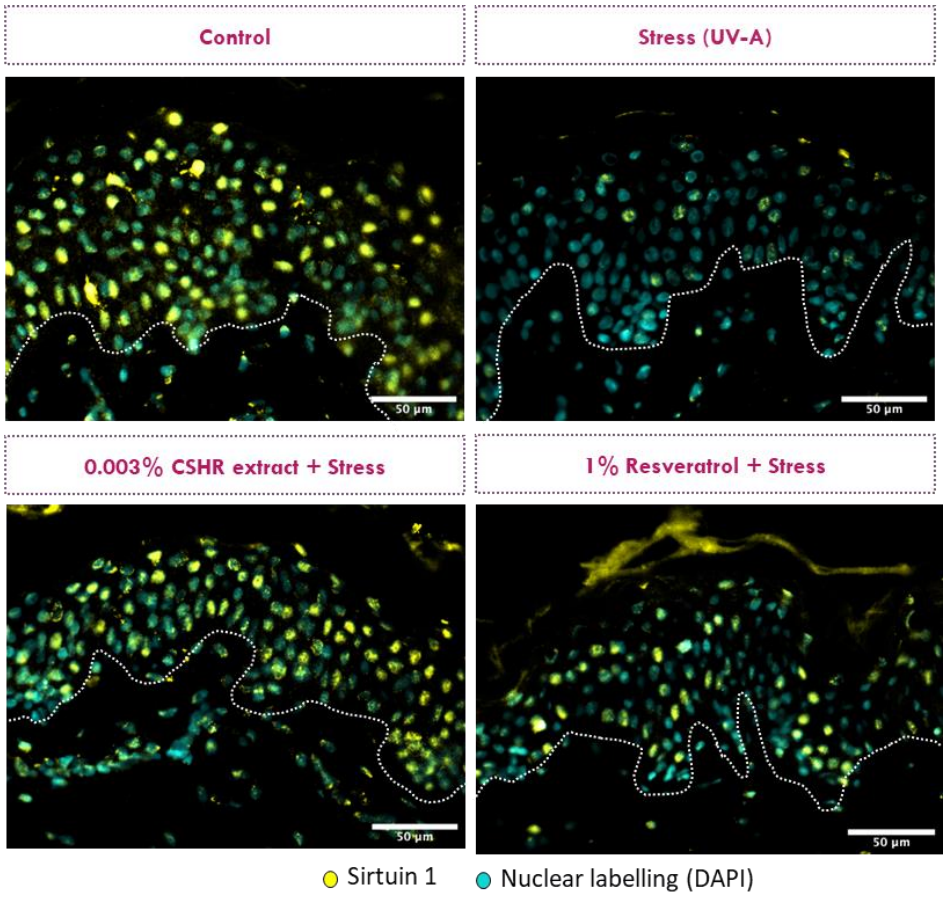

Figure 4: *In-situ* visualization of sirtuin-1 levels. The white dotted line separates the epidermis and the dermis.

Compared to the control, stress (UV-A) significantly decreased the sirtuin-1 levels. The presence of CSHR extract significantly counteracted (83% efficacy) stress-mediated decrease of sirtuin-1 levels, suggesting a protective property of longevity. The positive reference (1% Resveratrol) also protected the levels of sirtuin-1 upon UV-A irradiation with efficacy of 68%.

3.2.4. P16^{INK4A} *levels*

Compared to the control, the stress (UV-A) significantly increased the number of P16^{INK4A}-positive cells. The presence of CSHR extract significantly counteracted (89% efficacy) the stress-mediated increase of P16^{INK4A}-positive cells, suggesting anti-senescent properties.

3.2.5. Keratin-14 levels

In-situ detection of Keratin-14 levels (visualized in purple) was performed by epifluorescence microscopy (Figure 5). The stress (UVA) decreased the levels of Keratin-14. The presence of the CSHR extract counteracted the stress-mediated decrease in the levels of Keratin-14.

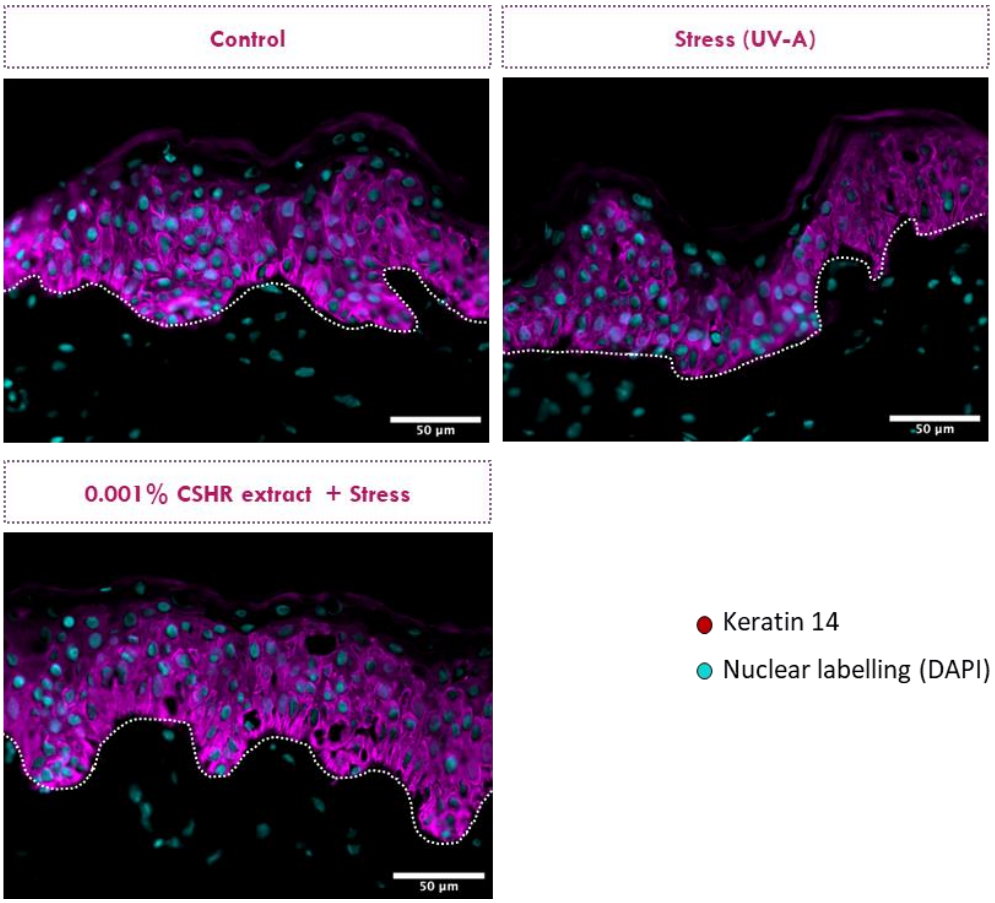

Figure 5: *In situ* visualization of K-14 levels. The white dotted line separates the epidermis and the dermis.

Compared to the control, stress (UV-A) significantly decreased the K-14 levels. The presence of CSHR extract significantly counteracted (95% efficacy) the stress-mediated decrease of Keratin-14 levels, suggesting a protective property of epidermal integrity and renewal.

3.2.6. TNF-α and IL-6 levels

Compared to the control, stress (UV-A) significantly increased the levels of the two inflammatory biomarkers (TNF-α and IL-6). The presence of CSHR extract significantly counteracted the stress-mediated increase of TNF-α (95% efficacy) and IL-6 (46% efficacy) levels, suggesting anti-inflammaging properties.

4. Conclusion

In today's society, the pursuit of longevity and well-being drives the demand for natural, sustainable skincare solutions. Hemp-derived compounds, particularly cannabisins, offer promising antioxidant, anti-inflammatory, and neuroprotective benefits. This study explored the potential of a cannabisin-rich extract from bioreactor-cultivated *Cannabis sativa* hairy roots to enhance longevity by stimulating key biological pathways, including proteostasis, autophagy, and senescence protection. The extraction was made using a microwave-assisted process to increase the content of cannabisin F by 46 times. If combined with the 100-fold increase in cannabisins achieved through hair roots technology, this extraction would enable the recovery of approximately 4600 times more cannabisin F in a CSHR microwave-assisted extract compared to a standard hemp seed extract.

Ex vivo studies involving human skin samples were performed for three concentrations – 0.001%, 0.003%, and 0.01% – showing remarkable protective effects on UV-A-induced carbonylation levels. We also demonstrated significant improvements in Sirtuin-1, Lamp2A, $P16^{INK4A}$, K-14, TNF-α, and IL-6 levels, even compared to resveratrol. These results suggest that this cannabisin-rich eco-extract from bioreactor-cultivated hemp offers a promising solution for promoting skin longevity, while ensuring respect for the planet and the biodiversity.

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