The Hair Tensegrity: applying an architecture-inspired concept to hair care

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ABSTRACT

Hair tensegrity is a concept based in the compensation and long-distance effects of tensional and compressional forces suffered by the scalp-hair due to complex interactions between the extracellular matrix compounds, skin cells, hair fiber elements and intercellular spaces. The maintenance of hair tensegrity is key for the health of both scalp and hair, although it is constantly threatened by external agents and ageing. In this paper we have analyzed the potential of an Encapsulated Cellular Oil (ECO) product based on the phyto-lipidic fractions of *Olea europaea var. silvestris* plant stem cell cultures on the preservation and boosting of all the elements involved in the maintenance of hair tensegrity in *in vitro*, *ex vivo* and volunteers *in vivo* trials. The results of the different assays show that the ECO has a strong antioxidant effect, avoids protein and hair fiber damage by different mechanism, penetrates the hair fiber protecting it from compounds release (e.g., hair dyes), increases hair diameter and strength, while increases cell proliferation, enhances various hair and scalp parameters in volunteers and reduces TEWL. Additionally, ECO has been revealed as an excellent booster of the production of different ECM compounds, including various types of collagens (III, XVII) and versican, also suppressing the degradation of proteins involved in cellular attachment. Altogether the results position ECO as a novel and effective multitarget treatment to maintain and boost scalp and hair health by an innovative hair tensegrity promotion approach.

Keywords: Hair Scalp; Tensegrity; Encapsulated Cellular Oil; Plant Stem Cell Culture; Phyto-Lipidic Fractions.

INTRODUCTION

Traditionally, the hair care market centered its attention exclusively and broadly on the hair shaft and ignored its complex structure and layers (medulla-cortex-cuticle) interactions. Further, the interrelation of hair fibers and scalp has been traditionally obviated, while nowadays revealed key for the proper activity of the entire tissue. Scalp fitness is essential for hair health **[1,2]**, and the relationship between scalp conditions and changes in composition of the emerging hair, the cuticle, capillary fragility, lack of shine, among others, has been clearly established. The skin of the scalp acts as anchor and support of the hair follicles, being the extracellular matrix central for this interaction and fundamental for the formation of healthy hair. These intrinsic and extrinsic interactions and forces applied in the tissue are governed by a continuum, build from the extracellular matrix elements and by the interaction of the cells that form the system (keratinocytes, fibroblasts, epithelial cells of the hair follicle, germ cells, trichocytes) and the intercellular spaces. Hair tensegrity corresponds to the biological engineering supporting and articulating the entire process **[3-9]**, being essential that the skin remains well cohesive, compact, elastic, and firm, for an adequate maintenance of it.

A key characteristic of the scalp is the large presence of hair follicles. These are constructed as an epidermal invagination in the papillary dermis, which generates the space and mold on which the living hair shaft is formed and grows, and which delimits and interconnects it on one side with the surrounding skin tissue of the scalp, and on the other side with the emerging hair fiber **[10]**. Thus, on the outside, the follicle is surrounded by a vitreous membrane, a layer of fibrous connective tissue that connects it directly with the cutaneous dermis. These elements, connected to each other, contribute to the cohesion and adequate formation in the composition and directionality of the hair. In the interior and regarding the concept of continuity, the cells that form the stem constitute a whole.

Hence, to guarantee the correct tensegrity of the tissues they must maintain certain viscoelastic properties based on the materials that composed them. Among these compounds three types are especially relevant, including: a network of fibrous proteins (different types of keratin intermediate filaments, collagens, etc.) which acts as support elements to stabilize tension or torsion, a network of anchoring proteins (versican, collagen 17, etc.) which provide a structural binding support in the extracellular matrix (ECM), and a system of intracellular matrixes that interconnects the fiber network though the hair shaft.

In this paper we have evaluated the effect of an Encapsulated Cellular Oil (ECO) product based on the phyto-lipidic fractions of *Olea europaea var. silvestris* plant stem cell cultures developed by *Vytrus Biotech S.A* on different tensegrity related parameters on scalp and hair fibers. Additionally other variables such as the antioxidant, antiglycation, anti-carbonylation and anti-desquamation effect of the ECO were evaluated in *in vitro*, *ex vivo* and *in vivo* assays to deeply characterize the properties of this innovative product.

MATERIALS AND METHODS

In vitro assays

Antioxidant enzymatic assay was tested by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging as previously described **[11, 12]** and using ascorbic acid as positive control.

Antiglycation enzymatic assay was evaluated by the quantification of the fluorescence intensity associated to Advanced Glycation End product (AGE) at an excitation wavelength of 370 nm and an emission wavelength of 440 nm (based on **[13]**) and using aminoguanidine as positive control.

Anti-protease enzymatic assay was based on the measurement of Kallikrein-5 (KLK5) activity. Enzymatic reaction was performed at RT in 100 mM NaH2PO4 buffer (pH 8.0) containing 0.25 μg/mL recombinant human KLK5, 100 μM of Boc-V-P-R-AMC Fluorogenic Peptide Substrate (R & D

Systems Inc.), and 1.1% DMSO at final concentrations. KLK5 (final 8.1 nM) was preincubated with test samples for 5 min, followed by the addition of peptide substrate. After incubating for 5 min, RFU was measured at Ex 380 nm/Em 460 nm. Leupeptin hemisulfate (42.1 μM) was used as a positive control.

The qPCR evaluation of *COL3A1* expression was carried out in Human Dermal Fibroblasts (HDF). They were seeded in triplicate in 6-well plates at a density of $3x10^5$ cells/well and were maintained for 24 hours, after which were exposed to ECO for 24h and analyzed by two-step RT-PCR **[14]**. Two genes were analyzed: *COL3A1* and Human *GAPDH* endogenous control.

Proliferation was evaluated in HDF cultures in 96-well plates at a density of 5.000 cells/well cultured for 24h. ECO was incubated during 24h and subsequently a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test was assayed **[15]**.

Ex vivo assays

Evaluation of hair shafts carbonylation

Antioxidant enzymatic assay was tested by 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging as previously described **[11, 12]** and using ascorbic acid as positive control.

Antiglycation enzymatic assay was evaluated by the quantification of the fluorescence intensity associated to Advanced Glycation End product (AGE) at an excitation wavelength of 370 nm and an emission wavelength of 440 nm (based on **[13]**) and using aminoguanidine as positive control.

Hair shafts were distributed in groups: control, stress, placebo and ECO. The group control and stress did not receive any product treatment. Hair shafts provided were gently treated with homogenous amounts of products for 10 minutes followed by 2 minutes of "massage". Then, hair shafts were dried and subjected to UV-A irradiation (LED source, emission peak at 365 nm, 84 J/cm2). The control group was not irradiated. Samples from each experimental group were sampled after the irradiation, cryo- preserved in Cryomatrix (OCT), snap-frozen into liquid nitrogen and kept at -80°C until analysis. The remaining material was stored at -80ºC. Sections of 3 μm of thickness were obtained using a cryostat (*Leica*) for the sagittal view. The imagining of the hair lateral view was realized along the longitudinal axe of hair shafts. Carbonylated proteins were labeled using a fluorescent probe (Ex = 647 nm / Em = 650 nm) functionalized with an aminooxy moiety to specifically bind carbonyl groups. Fluorescent images were collected with an epi-fluorescent microscope (*ThermoFisher, Evos M5000*) and analyzed with ImageJ software. Image collection for the different conditions was achieved using identical conditions of acquisition (40X objective). Representative images per condition and point of view (sagittal or lateral) are shown in the RESULTS section.

Protection of hair colour in dyed hair

Caucasian hair tresses from different donors (22cm long and 5cm wide), underwent a prewashing process consisting of wash with neutral shampoo, rinse with water for 2 min and dry with towel and hair dryer. Then tresses were immersed in ECO or placebo for 10 min, massage 2 min and dry naturally. The treated tresses were dyed using 1 part oxidizing cream 30 vol. 1 part hair dye with an incubation time of 40 min, were washed with running water at 37± 1ºC and with 2g of CTC neutral shampoo for 30 seconds. Later a shampoo with acid pH was applied to close the cuticles and water was eliminated by towel and hair dryer. Luminosity and colour variation were evaluated from images taken using a spectral range of 350 – 750 nm, a geometry of d/8º, a light of D65 and with an observer angle of 10º.

Evaluation of penetration into hair cortex

The penetration of ECO and placebo into the hair cortex was evaluated using Leica TCS-SP8 confocal microscope, with an *Objective Plan Apochromatic* 63x (NA 1.4) and a 1.75 optical zoom. The capture

parameters for confocal microscopy are: - 1024 x 1024 pixel format - λex(max) excitation fluorescence mode: 488nm Argon laser - fluorescence detection interval for *BODIPY™ FL C12 Sphingomyelin*: 500- 585nm. Based on the series of images of the slices obtained by cryostat and the MIP, Extended Focus projections were obtained using the *Stack Arithmetic-Sum function* of *Image/Fiji* analysis software package (*Universal Packaging, PA*, (USA). The resulting images are more of 16 bits. In the images obtained, the levels of greys are proportional to the quantity of fluorescence detected, so, given the study conditions, they are also proportional to the amount of compound in each sample.

Evaluation of hair diameter and strength increase

Hair tresses were immersed for 10 min in ECO and placebo, massage for 2 min and dry naturally, after which diameter was determined by *Hand USB Digital Microscope* images. Strength was determined by measuring initial hair strength, as the maximum force (mN/µm) that each tress could withstand while being stretched before breaking. Then, 5 tresses were soaked in placebo lotion and 5 in 1% ECO, after what tresses were left to dry naturally for 12 hours (leave-on application). Finally, once the tresses had been allowed to dry naturally, a hair straightener (230ºC) was used on each of the 10 tresses, and then the hair strength was measured again and compared with the initial measurement.

***In vivo* trials**

***In vivo* assay I**

Single-center double blind study was carried out on 20-woman volunteers with ages ranging from 19 to 70 years old and dry hair. The study was conducted according to the standard operating procedure of *Centro de Tecnología Capilar S.L.* and in compliance with the regulations established in "*Guía para investigaciones con seres humanos*" (Guidelines for Research on Human Beings) and the guidelines of the Scientific Committee on Consumer Safety (SCCS). To start the trial, each volunteer went to the C.T.C. facilities, where microphotography of the scalp was taken by microcamera and scalp and hair was evaluated by technician. At volunteers' homes, they divide their head into two-part according to **Figure MM1,** and apply about 4-5ml of the corresponding product (ECO lotion or placebo) in the scalp and massage with fingertips to facilitate absorption. The hair of each head part was sprayed with about 15ml of the product and massage to facilitate absorption. The product was not rinsed after application in any case. In the visit to C.T.C. facilities microphotographs of the scalp were taken and evaluations by technicians were performed. Evaluation was repeated after one and four product applications following the same protocol. The microcamera is a professional diagnostic piece of equipment consisting of a probe for exploring the scalp and the software for capturing and displaying images. The microcamera's function is to allow a direct, magnified view of a scalp area to observe its state: whether greasiness, dandruff or redness are present, etc. When the images obtained are compared, interesting graphic evidence of the effectiveness of the products tested is obtained. Additionally, several parameters regarding hair and scalp were evaluated by the experienced technicians in each volunteer and area. The parameters include easiness to detangling, combability, softness, manageability, volume, hydration, shine and natural movement. Results were processed and expressed as percentage of improvement if comparing with T=0.

Figure MM1. Right and left head division's example

In vivo assay II

A second double-blind, placebo-controlled in vivo trial was carried out during 28 days on a panel of 15-woman volunteers with ages ranging from 19 to 70 years old. The lotions were applied twice daily following the same protocol than in in vivo assay I. After those 28 day of application, a total of 10 hairs per volunteer were carefully plucked to recover the integrity of the hair follicle for subsequent analysis of the gene expression of two hair anchoring proteins (versican and collagen 17). Within this same trial, an analysis of the scalp TEWL was also performed. Gene expression was evaluated by qPCR (two-step RT-PCR **[14]**, while the scalp TEWL was analyzed with a *Nano Tewameter*® (*Courage & Khazaka*).

Statistics processing

The unpaired t-test and one factor analysis of variance (ANOVA) with Bonferroni-Dunn´s correction was performed to assess differences in all studied parameters. P-values ≤ 0.05 were statistically significant.

RESULTS

***In vitro* assays**

The main factors shaping the potential of ECO to protect and regenerate the scalp and hair were evaluated in different *in vitro* assays. Representative tests regarding the antioxidant effect, protein protection, reduction in desquamation though KLK5, collagen production and cell proliferation were selected.

In the case of the antioxidant activity evaluation (AA), statistically significant differences were observed between the samples and the untreated control, being higher than 80% for both ECO concentrations (**Figure 1A**). Data from the anti-glycation activity assay show a linear increase in ECO activity surpassing in the case of 10% sample the 50% antiglycation activity shown by the positive control (4mM aminoguanidine) (**Figure 1B**). KLK5 assays reported that all ECO samples (5% and 10%) strongly inhibited the activity of the protease, ranging from -36% to -47% reduction and suggesting ECO's potential against desquamation (**Figure 1C**). Collagen III production by HDF was also induced 6-fold times by the exposure to 0.1% ECO (**Figure 1D**), while the proliferation of this cell line was increased between 21% to 51% if compared with the untreated control.

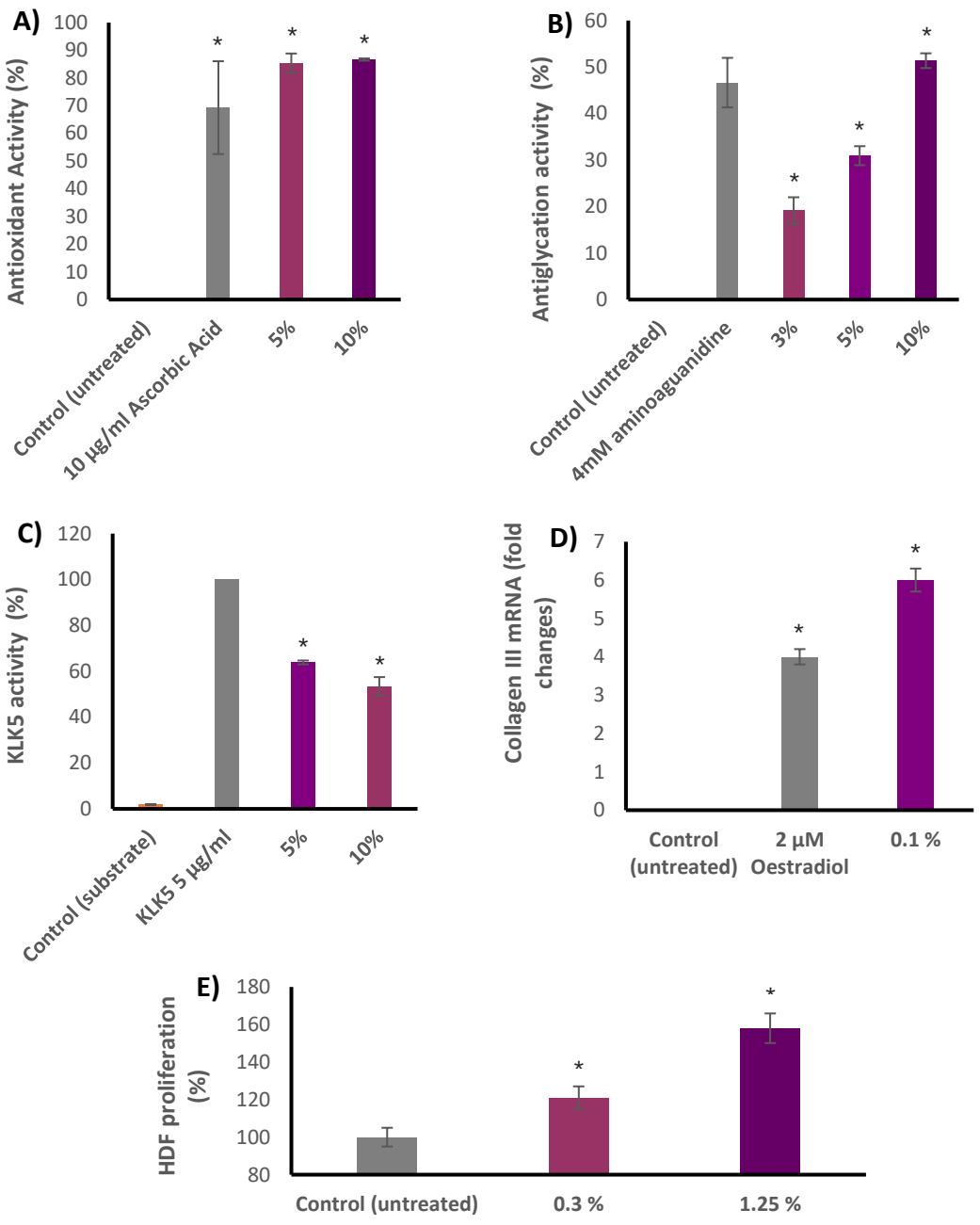


Figure 1. ***In vitro*** **effects of ECO treatment**. **A)** Antioxidant activity displayed by ECO and measured by DPPH. Ascorbic acid is used as positive control. **B)** Reduction in the relative content of AGEs by ECO. Aminoguanidine is used as positive control. **C)** Inhibition of KLK5 activity at different ECO concentrations. KLK5 + substrate is used as control and corresponds to 100% activity. **D)** Increase in type III collagen expression in HDF after treatment with ECO. qPCR validation of *COL3A1* expression in HDF cells. Estradiol was used as control **E**) Increase in HDF cell proliferation after treatment with ECO. Proliferation was evaluated in HDF cells treated with different concentrations of ECO by MTT. The results are expressed in the increase of cell proliferation. The vertical bar represents the mean and standard error of the mean for each condition and in every graph. The means in each bar with an asterisk are significantly different if compared with the untreated control (at P≤0.05).

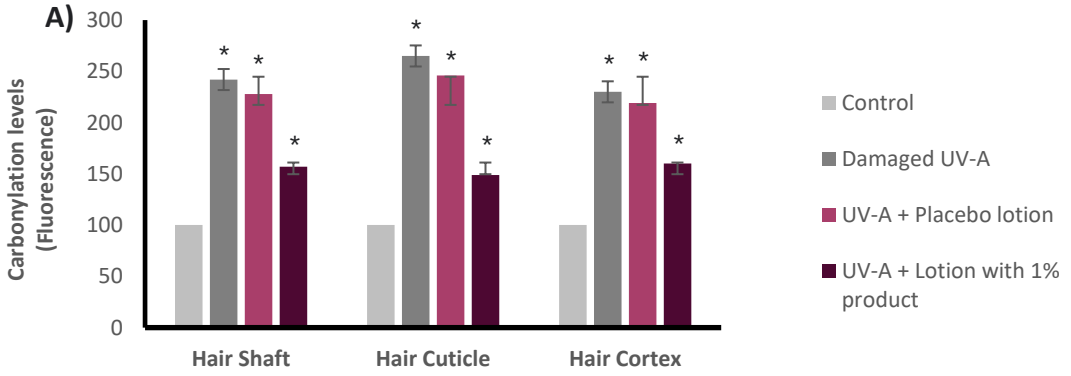
Ex vivo assays: efficacy of hair fiber

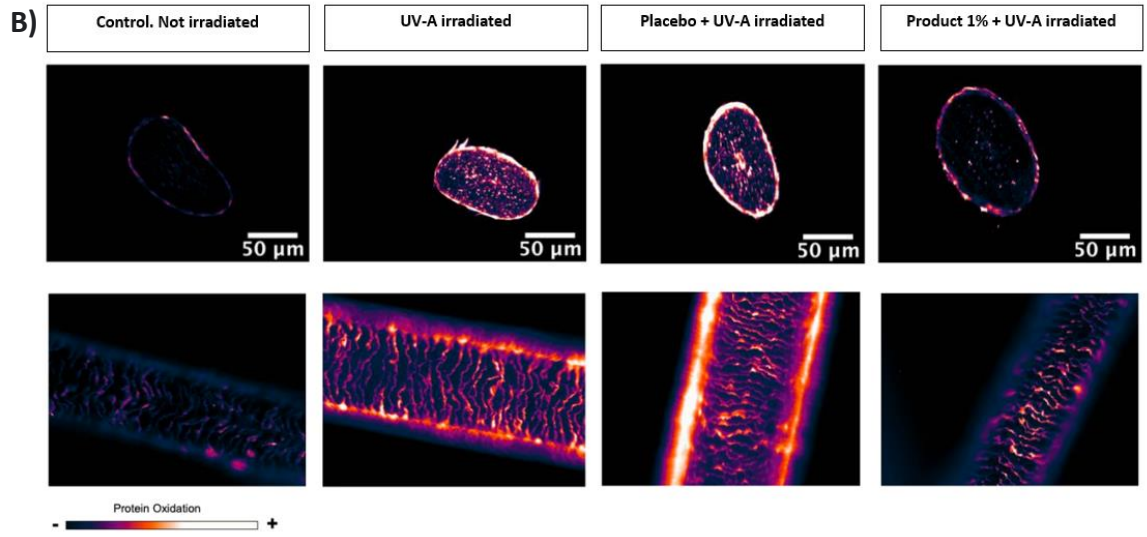
The *in vitro* assays carried out demonstrated that ECO triggers protective and regenerative effects in cells and models potentially replicable into the scalp. However, additional assays were necessary to

validate the direct effect of ECO on hair fibers. Different assays were selected with this purpose, including the protection against different stressors, the nourishing of the fibers, and the improvement of different fiber parameters. Altogether these assays expanded the knowledge about the potential of ECO for hair treatment and allowed to go further and articulate *in vivo* trials that integrated the extensive discoveries made in the *in vitro* an *ex vivo* testing.

Protection of hair proteins from UVA-induced carbonylation and high temperatures

UVA-induced carbonylation and hair fiber structure maintenance after thermal treatment was evaluated. ECO's hair protector effect from UVA-induced carbonylation was measured with a pre-treatment of 10 min with each lotion + 2 min massage, then UV-A (84 J/cm2), followed by incubation with fluorescent probe (binding carbonyl groups) and microscope image acquisition. The product demonstrated to protect the hair shaft by a mean of 60%, the hair cuticle by 70% and the hair cortex by 54% after only one application of ECO (**Figure 3A**). Also, microscope images showed higher fluorescent signal (corresponding to higher carbonylation) in the case of not treated and placebo samples (**Figure 3B**).




B)
Control. Not irradiated
UV-A irradiated
Placebo + UV-A irradiated
Product 1% + UV-A irradiated
50 µm
50 µm
50 µm
50 µm
Protein Oxidation
-
+

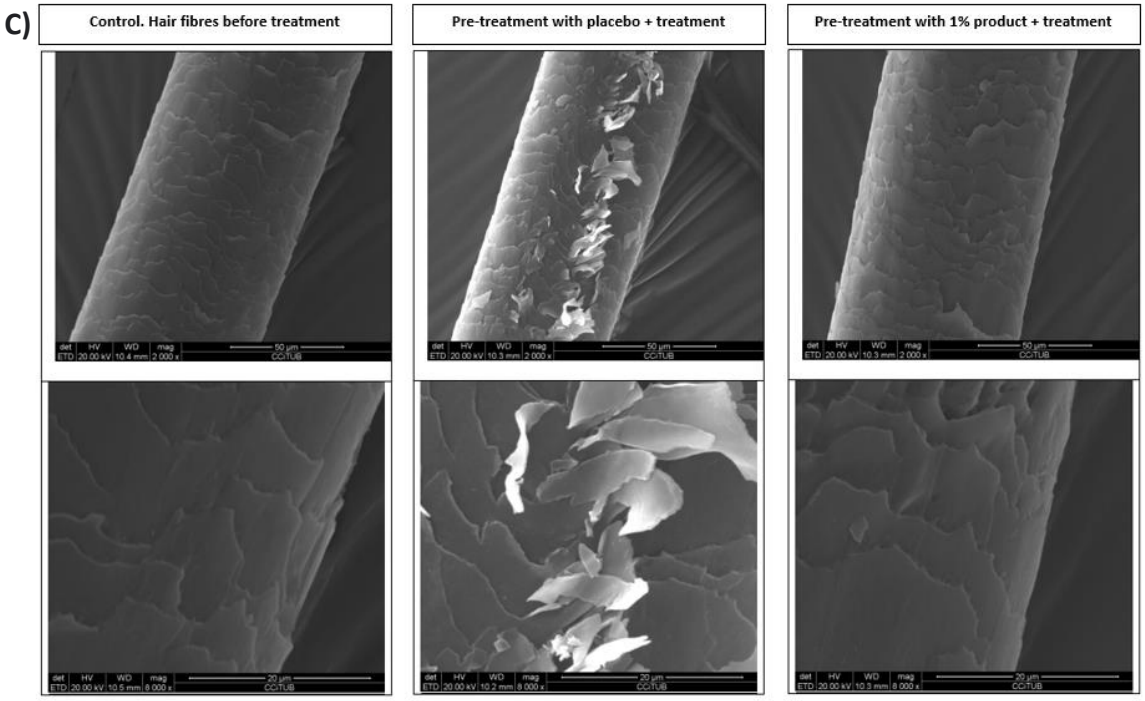
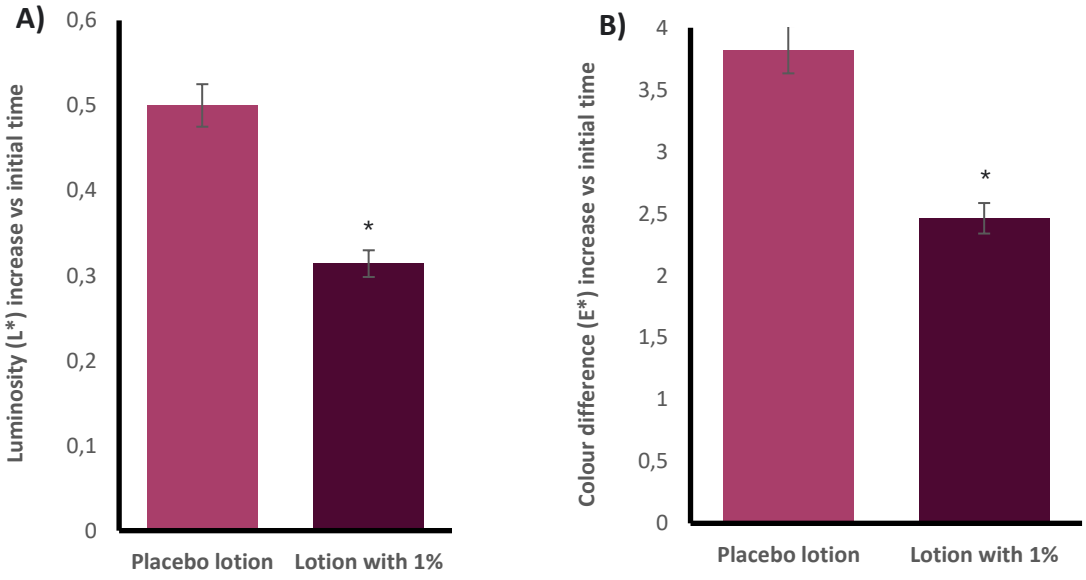

C)
Control. Hair fibres before treatment
Pre-treatment with placebo + treatment
Pre-treatment with 1% product + treatment

Figure 6. **Protection of hair fibers A)** Reduction of carbonylation after treatment with ECO. Graph representing the carbonylation levels evaluated though fluorescence signal of hair fibers after UV-A irradiation. Control corresponds to not irradiated hairs and UV-A irradiated without protection. The vertical bar represents the mean and standard error of the mean for each condition. The means in each bar with an asterisk are significantly different if compared with the control (at P≤0.05). **B)** Representative images corresponding to the *In situ* visualization of oxidized proteins, sagittal view and cuticle view (lateral). The fluorescence emission signal for carbonylated proteins was obtained by using a fluorescent probe that absorbs and emits light at different wavelengths (647/650 nm). Bars correspond to 50 µm as indicated. **C)** Reduction of thermal induced damage after treatment with ECO. Representative images corresponding to the electron microscopy visualization of hair fibers before thermal treatment (T ≥ 230ºC), and after pre-treatment with placebo or 1% ECO and thermal treatment. Bars correspond to 50 µm and 20 µm as indicated.

Additionally, a test to obtain representative images from scanning electron microscopy after a thermal treatment (T ≥ 230ºC) was carried out to determine the efficacy of the ECO against heat damage. It was observed that the hair fibers treated with 1% ECO before the thermal treatment showed less damage than those treated with placebo (**Figure 3C**). The damaged fibers showed desquamation and broken areas all around its structure.

Protection of hair colour in dyed hair

ECO showed a strong activity against dye colour loss and variation (**Figure 4A)**. In both cases, the application of ECO enhances the properties of the dyed hair, preventing the dye colour loss with an average of 37% (luminosity) and the colour variation with an average of 36%. **Figure 4B** shows some representative images of the hair strands used in the assay.



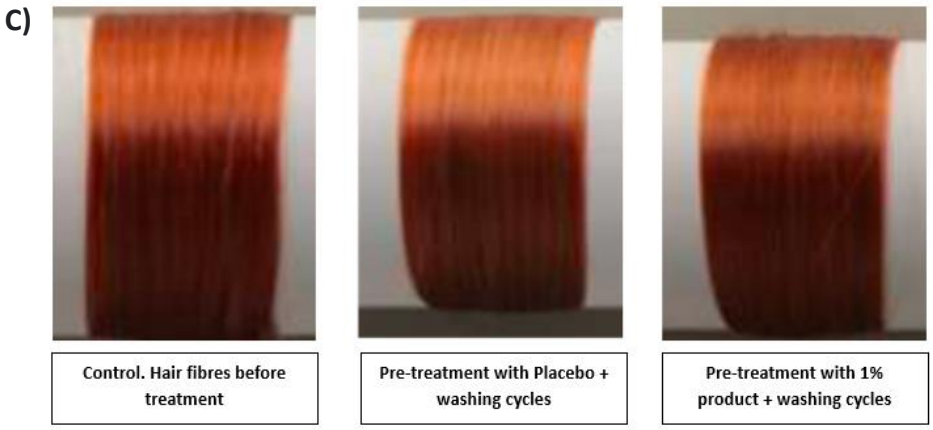


Figure 4. **Protection of dyed hair**. **A**) Graphs representing the variation in hair dye loss and colour variation in hair strands before 12-cycles wash treatment and after the pre-treatment with placebo and 1% ECO and the wash cycles. The vertical bar represents the mean and standard error of the mean for each condition. The means in each bar with an asterisk are significantly different if compared with the placebo (at P≤0.05). **B)** Representative images corresponding to hair strands used in the assay. From left to right: hair strands before 12-cycles wash, placebo pre-treated strands after wash treatment, and 1% ECO pre-treated strands after wash treatment.

Penetration of ECO into the hair cortex

To evaluate the capability of ECO to efficiently deliver into the hair fibers, and determine its potential to affect the hair cortex, individual hairs were exposed to placebo and 1% ECO and images were obtained. Based on the series of images of the slices obtained by cryostat and the MIP, Extended Focus projections were obtained using the Stack Arithmetic-Sum function of Image/Fiji analysis software package (Universal Packaging, PA, (USA). The resulting images were more of 16 bits. In the images obtained, the levels of greys are proportional to the quantity of fluorescence detected, so, given the study conditions, they were also proportional to the amount of compound in each sample. Because of this, if an area shows higher levels of greys, it means that it contains more fluorescent compounds. The quantification of levels of fluorescence in each sample was calculated based on the values obtained in the Image J/Fiji image analysis package: the average intensity value (average of the pixels corresponding to the hair) and the integrated intensity (area x average intensity) was calculated.

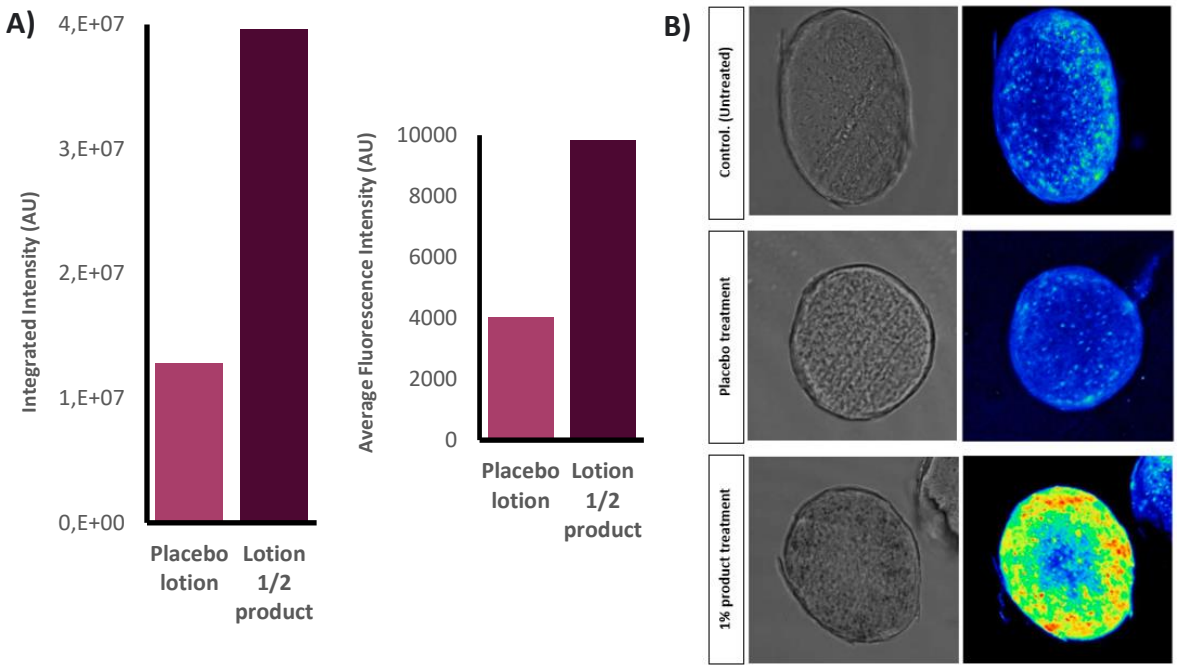


Figure 5. **Penetration into hair fibers**. **A**) Graphs representing the variation in hair dye loss and colour variation in hair strands before 12-cycles wash treatment and after the pre-treatment with placebo and 1% ECO and the wash cycles. The vertical bar represents the mean and standard error of the mean for each condition. **B)** Representative images corresponding to hair strands used in the assay. From left to right: hair strands before 12-cycles wash, placebo pre-treated strands after wash treatment, and 1% ECO pre-treated strands after wash treatment.

In the images, there was a clear increase in fluorescence intensity detected in the hair samples treated with ECO and marker, while the images of hair samples treated with placebo and marker are similar to the control hair samples (**Figure 5A**). To compare the results obtained for the two products, ECO and placebo an image of natural human hair (without treatment) was taken as a reference point of this analysis. Subsequently, the quantification of the transverse section of the hair (20 μm thick) corresponds to 30 fields of each of the lotions. The average intensity value (average of the pixels corresponding to the hair) and intensity integrated (area x medium intensity). Quantification indicates that ECO lotion has a medium intensity (9830.2 arbitrary units(a.u.)) and an integrated intensity (39766335.8 a.u.) far superior to placebo lotion (**Figure 5B-C**). These quantifications corroborated the visual results and showed a 2.45- and 3.1-fold increase of fluorescence if compare the ECO with the placebo or untreated samples respectively.

Hair diameter increase

ECO effect on hair diameter was also evaluated. The hair diameter was measured by micro-camera before and after treatment with a placebo lotion or with a lotion containing 1% ECO. The protocol was based on pre-wash the tresses with neutral shampoo 1 min + 2 min rinse-off and allow to dry, initial hair diameter measurement with the micro-camera followed by treatment of 10 min with each lotion + 2 min massage and allow to dry, and finally, measurement of final hair diameter

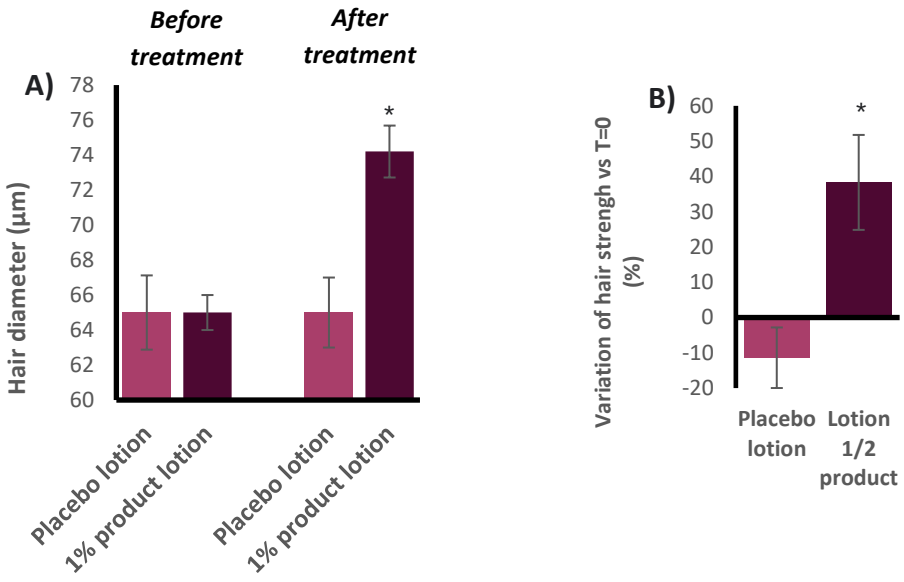


Figure 6. **Changes in hair diameter and strength. A)** Increase in hair diameter after treatment with ECO. Graphs representing the variation in hair diameter after the treatment with placebo 1% ECO lotions. The vertical bar represents the mean and standard error of the mean for each condition. **B)** Increase in hair strength after treatment with ECO. Graphs representing the variation in hair strength before and after pre-treatment with placebo or 1% ECO lotion and thermal treatment. The vertical bar represents the mean and standard error of the mean for each condition. The means with an asterisk are significantly different if compared with the placebo (at P≤0.05).

The results showed an average increase in hair diameter of 14% when comparing the 1% treated samples with the placebo (**Figure 6A**). The 14% increase in diameter corresponds to an average increase of 9.2µm.

Increase in hair strength

The treatment with 1% ECO increased the strength of the hair fibers an average of 38% if compared with T=0 and a 50% if compared with the placebo treated samples (**Figure 6B**). In the case of the placebo samples, a reduction in strength after the treatment was observed.

***In vivo* assays**

Once demonstrated the strong effect of ECO on the *in vitro* assays, proving their effects on the scalp cells and environment, as well as the potential on hair fiber in *ex vivo* assays, a series of *in vivo* trials were designed to evaluate and demonstrate the efficacy of the active ingredient on the volunteers' scalp and hair appearance.

***In vivo* assay: I**

The first *in vivo* trial was performed on a 20-volunteer panel aged 19-70 years old. The study was double-blind, and placebo controlled. The assay was carried out during the pandemic in volunteers

with sensitive scalp. Several parameters were analyzed by trichologists to see the performance of a cream containing the active ingredient at 0.5% dosage.

Evaluation of hair conditioning

The first marker to be analyzed was the hair conditioning and how the ingredient beautifies the hair through a trichologist analysis. As shown in the **Figure 7A**, there was a significant improvement of the hair, showing an enhanced softness, volume, and hydration after only one application and demonstrated an increase of the effects after four applications (**Figure 7B**).

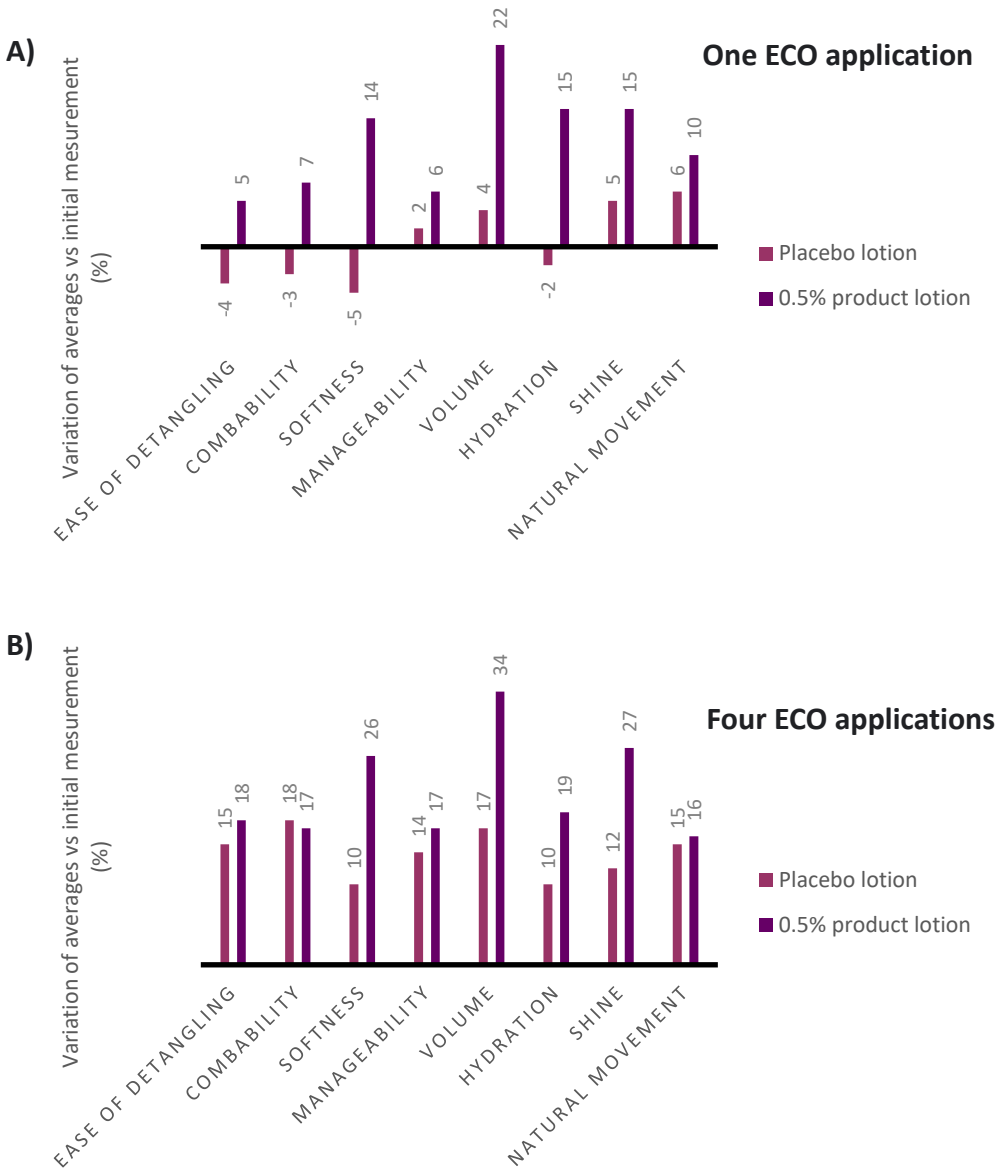


Figure 7. **Variations in hair conditioning parameters**. **A)** Graphs representing the average variation in different hair parameters after one application of placebo or 1% ECO lotion. The vertical bar represents the mean and standard error of the mean for each condition. **B)** Graphs representing the average variation in different hair parameters after four applications of placebo or 1% ECO lotion. The vertical bar represents the mean for each condition.

Reduction of scalp redness

Reduction on scalp redness was also evaluated by trichologist analysis demonstrating that ECO reduced the scalp redness after four rounds of treatment. A reduction of 68% was observed comparing the ECO group before and after the trial while a reduction of 32% when comparing placebo and ECO group after the four rounds of treatment (**Figure 8**).

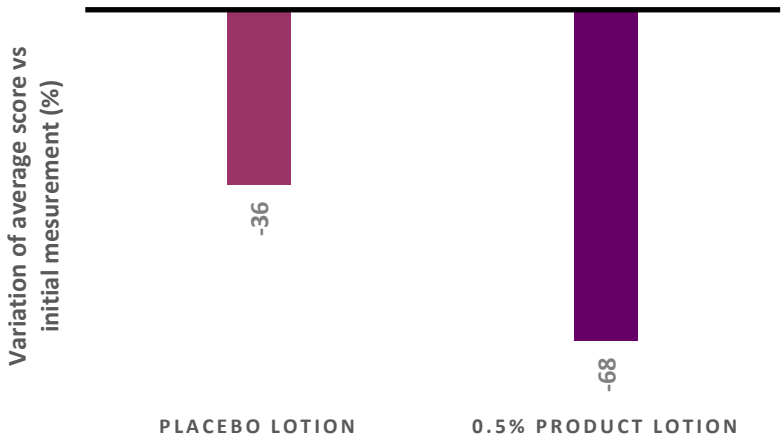


Figure 8. **Reduction in scalp redness**. Graphs representing the average variation in scalp redness before and after four rounds of treatment with placebo or 1% ECO lotion. The vertical bar represents the mean for each condition.

Visual evaluation of redness by micro-camera photography

When volunteers' scalps were evaluated using the micro-camera system, the result corroborated the trichologist's visual observation, with a large proportion of the scalps treated with 1% ECO showing a clear reduction of its redness and area inflammation. **Figure 9** shows representative images of different volunteers and their variation on both parameters after 1 or 4 applications of the placebo or 1% ECO.

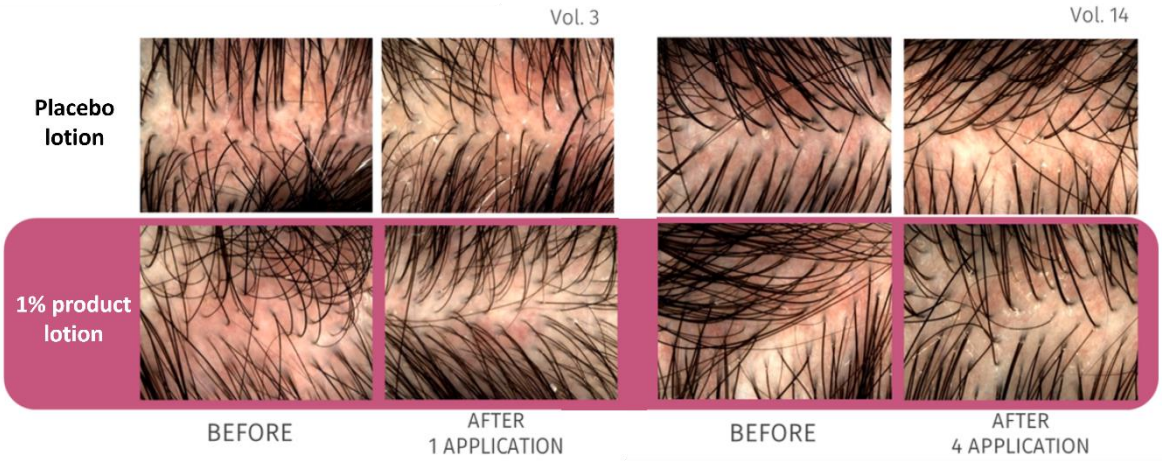


Figure 9. Micro-camera evaluation of the reduction in scalp redness. Representative images of the scalp of different volunteers treated with the placebo or 1% product. The images on the left side correspond to 1 application of ECOs, while the images on the right correspond to 4 applications of ECO.

In vivo assay: II

A second double-blind, placebo-controlled *in vivo* trial, carried out during 28 days on a panel of 15 volunteers, the effect of a lotion containing 1% ECO was analyzed. The lotions were applied twice daily on each half of the volunteer's scalp, analyzing subsequently *versican* and *collagen 17A* expression and TEWL levels.

Increase in *versican* and *collagen 17A* gene expression and TEWL

Versican and collagen 17 are two key proteins of the Extracellular Matrix (ECM). Versican is a proteoglycan which, together with the non-fibrillar collagen 17A, helps maintain the tissue shape, thus keeping the 3D skin structure. This makes these two markers very interesting when analyzing the capacity of 1% ECO to strengthen the scalp. Therefore, hair follicle biopsies of 10 volunteers were analyzed before starting the assay. The initial levels of *versican* and *collagen 17A* were determined by qRT-PCR. At the end of the test, the qRT-PCR analysis was carried out a second time, in order to compare the results.

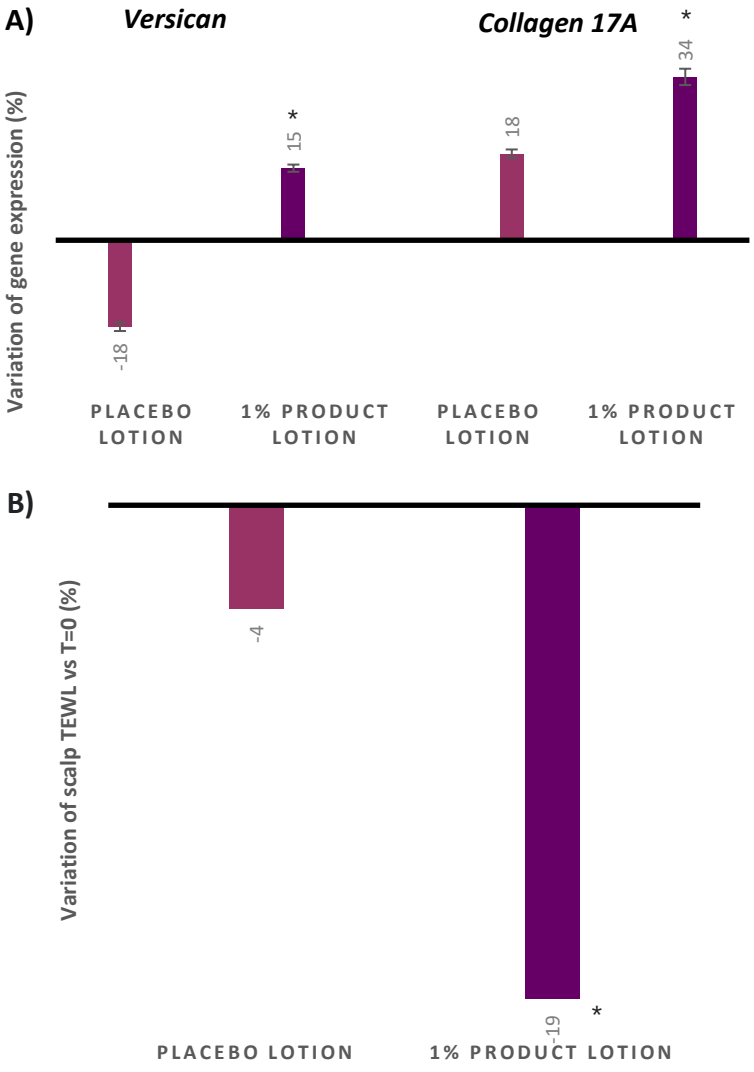


Figure 10. **Results from the in vivo trial II. A)** Variation on *versican* and *collagen 17A* gene expression after treatment with 1% ECO lotion. Graph representing the variations in *Versican* and *Collagen 17A* in volunteers scalp evaluated by qPCR after treatment with placebo or 1% ECO lotion. The vertical bar represents the mean and standard error of the mean for each condition. The means with an asterisk are significantly different if compared with the placebo (at P≤0.05). **B)** Variation on TEWL after treatment with 1% ECO lotion. Graph representing the variations in TEWL in volunteers scalp evaluated by Nano *Tewameter®* (*Courage & Khazaka*) after treatment with placebo or 1% ECO lotion. The vertical bar represents the mean and standard error of the mean for each condition. The means with an asterisk are significantly different if compared with the placebo (at P≤0.05).

The analysis showed that 1% ECO induced the expression of the *vesican* and *collagen 17A* genes, with an average of 15% and 34% respectively if compared with the initial conditions and 33% and 16% if compared with the placebo group. Surprisingly, the results indicated that the placebo lotion decreased the expression of v*ersican*, while increased the expression of the *collagen 17A*. Taken together the results demonstrated that the 1% ECO lotion stimulated production of key anchoring elements of the scalp matrix.

Regarding the TEWL, ECO triggered a reduction of 19% of TEWL if compared with the initial testing while placebo only reduced 4% (**Figure 10B**).

DISCUSSION

Several assays have been used to determine the potential of ECO to revitalize the scalp and hair shaft in *in vitro* assays, *ex vivo* hair samples and trials with volunteers.

The *in vitro* assays showed the potential of the product as antioxidant treatment, also having a strong effect on the protection against protein modification, desquamation, cell proliferation and ECM components production. These *in vitro* results started to point the potential of the product to boost tensegrity, because demonstrated that the product could potentially affect the some of the tissue components involve in this process: structural proteins (by protection or promotion), connection proteins (by avoiding degradation) and cells (by increase of their proliferation).

Further, the effect of ECO was also challenged in a series of *ex vivo* assays, with the aim of demonstrating the direct effect of the product on the hair fibers.

Protein carbonylation is a harmful irreversible oxidative protein modification, considered as a major hallmark and a reliable biomarker of oxidative stress. In this light, the protective effect of the pre-treatment with ECO on hair fibers after UVA irradiation was evaluated, revealing that ECO displays a strong protective effect against carbonylation. This direct protection against the modification of hair fiber elements could be linked to the strong antioxidant effect exhibited by ECO in the *in vitro* assays and could suggest that ECO generates an antioxidant environment that can act on multiple targets.

The protection of colour in dyed hair was also evaluated in *ex vivo* assays, being the color fastness a major concern for consumers and manufacturers. Repeated washes of dyed hair have a noxious effect on their luminosity while inducing colour variation. This fact is determined by the tendency of hair dye to dissolve in water and leach from the hair shaft **[16]**. Products that help dyed hair fibers to maintain their colour characteristics without the need of potentially toxic substances are constantly

seeked by consumers and industry. Product tests demonstrated that ECO used as a pre-treatment before several washing cycles enhanced the properties of dyed hair fibers, preventing the dye colour loss. The results pointed out that the effect of ECO on hair was not only due to its excellent antioxidant activity and the protection against hair disruption, beyond it has a direct effect protecting the hair fiber from external aggression (in this case the washing) while avoiding the release of internal elements into the environment. These effects on element release could be linked to the fact that ECO could attach to the hair surface and enter in the inner structure as described.

Besides, and considering the penetration capacity of ECO, its potential to modify the hair structure was evaluated. Surprisingly, the treatment with ECO increases hair diameter. Two of the possible hypotheses for this thickening, among others, are that the penetration of ECO in the inner part of the fiber as well as its surface protection increase the diameter by incorporation of new material to the fiber or by the reduction in water and structural elements loss. Obviously, a combination of multiple mechanisms could be possible due to the multimodal effect of ECO.

To ensure that the modification of the fiber diameter and reduction of element release was not detrimental for the hair strength, this parameter was evaluated in *ex vivo* assays. Hair fibers were pre-treated with ECO or placebo and subjected to a head shock and a hair strength evaluation. ECO treated group induced an increase of the hair fiber strength while placebo reduced. These results suggested that no detrimental effect on the hair fiber characteristics was generated by ECO.

With different evidence regarding the effect of ECO obtained during the *in vitro* and *ex vivo* assays, we carried out a series of trials with volunteers to validate the results in scalp-hair context. Two trials were developed: a) the evaluation of hair conditioning and reduction of redness, and b) the determination of the levels of versican, collagen and the variation on trans epidermal water loss (TEWL).

All the hair conditioning parameters evaluated revealed an improvement after the treatment with ECO, supporting in an *in vivo* context all the results shown in previous assays. Among the different parameters measured, the increase in hair volume and hydration related directly with the results of the hair diameter evaluation and protection of hair element loss. The *in vitro* A) also revealed a strong capacity of ECO to reduce scalp redness and area inflammation. These results could be linked to the repressive effect of ECO on kallikreins (KLKs) revealed during the in vitro assays. KLKs activity has been linked to the enhancement of different skin pathogenesis by its implication on inflammation, exacerbating erythema, desquamation **[17,18,19]** and potentially redness.

The results of the second in vivo trial B), showed an increase in the expression of *versican* and *collagen 17A* genes, two key structural molecules maintaining the well-functioning of the scalp. These molecules have been described as anchoring proteins that functions as a cell-matrix adhesion molecules through stabilization of the hemidesmosome complex **[20]**, while are highly expressed in hair follicle stem cells (HFSCs), are required for the maintenance not only of HFSCs but also of melanocyte stem cells (MSCs) **[21]** and the connection with hair shafts **[22,23,24]** by acting as components or partners of anchoring complexes **[20**,**25]**.

In this specific case, there was suggested a direct connection with the results of the *in vitro* tests, that has already proven that ECO increases the production of structural proteins such as collagen III. Finally, the TEWL was evaluated in the scalp, revealing a reduction when scalp was exposed to ECO. This factor could be linked to the general improvement of the scalp and hair matrix.

This paper describes a novel hair and skincare product with a synergistic combination of activities that trigger a holistic effect in the scalp-hair relationship, inducing and improving most of the hallmarks of scalp and hair damage or ageing.

CONCLUSIONS

The deep characterizations of ECO activity on individual cells, isolated hair fibers, volunteer's scalp and hair have demonstrated the potential of the product in the maintenance of hair tensegrity by affecting several biological processes in a multimodal approach. ECO has been revealed as a strong antioxidant treatment with a potent activity on protein protection against stress modifications (glycation, carbonylation, etc). Further, ECO's activity on human hair fibers has proven excellent in increasing their diameter and resistance, while stimulating the production of matrix compounds such as collagen in *in vitro* cells. In volunteer trials, ECO proved their potential to improve several hair and scalp parameters, reduce redness and TEWL, and enhance the extracellular matrix composition and hair anchoring in the scalp by promoting the production of vesican and collagen 17. Altogether the results demonstrated that ECO is a novel and perfect treatment to maintain and boost scalp and hair health by an innovative tensegrity promotion approach.

BIBLIOGRAPHY

[1] Tajran J, Gosman AA (2020) Anatomy, Head, and Neck, Scalp. Publisher: StatPearls Publishing. Book available online. https://www.ncbi.nkm.nih.gov/books/NBK551565

[2] Trüeb RM, Henry JP, Davis MG, Schwartz JR (2018) Scalp conditions impact hair growth and retention via oxidative stress. Int J Trichology. 10: 262-270

[3] Ingber DE. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. J Cell Sci 1993; 104: 61113–27.

[4] Ingber DE, Dike L, Hansen L (1994) Cellular tensegrity: exploring how mechanical changes in the cytoskeleton regulate cell growth, migration, and tissue pattern during morphogenesis. Int Rev Cytol. 150: 173–224.

[5] Ingber DE (1997) Tensegrity: the architectural basis of cellular mechanotransduction. Annu Rev Physiol. 59: 575–99.

[6] Silver FH, Siperko LM, Seehra GP (2003) Mechanobiology of force transduction in dermal tissue. Skin Res Technol. 9: 3–23.

[7] Quatresooz P, Hermanns-Leˆ T, Ciccarelli A (2005) Tensegrity and type I dermal dendrocytes in acromegaly. Eur J Clin Invest. 35: 133–9.

[8] Quatresooz P, Hermanns JF, Paquet P, Pie´rard GE (2006) Mechanobiology and force transduction in scars developed in darker skin types. Skin Res Technol. 12: 279–82.

[9] Quatresooz P, Uhoda E, Pie´rard-Franchimont C, Pie´rard GE (2006) Cellulite histopathology and related mechanobiology. Int J Cosmet Sci. 28: 207–10

[10] Murphrey MB, Agaewal S, Zito PM (2022).Anatomy, Hair. Publisher: StatPearls Publishing. Book available online. https://www.ncbi.nlm.nih.gov/books/NBK513312/

[11] Kang YY, Kim JY, Song J, Mok H (2019) Enhanced intracellular uptake and stability of umbelliferone in compound mixtures from *Angelica gigas* in vitro. Journal of pharmacological sciences. 140: 8-13

[12] Ozyürek M, Bektaşoğlu B, Güçlü K, Apak R (2008). Hydroxyl radical scavenging assay of phenolics and flavonoids with a modified cupric reducing antioxidant capacity (CUPRAC) method using catalase for hydrogen peroxide degradation. Analytica chimica acta. 616: 196–206.

[13] Lee C, Yim MB, Chock PB, Yim HS, Kang SO (1998). Oxidation-reduction properties of methylglyoxal-modified protein in relation to free radical generation. The Journal of biological chemistry. 273:25272–25278.

[14] Wacker MJ, Godard MP (2005). Analysis of one-step and two-step real-time RT-PCR using SuperScript III. Journal of biomolecular techniques : JBT. 16:266–271.

[15] van Meerloo J, Kaspers GJ, Cloos J (2011) Cell sensitivity assays: the MTT assay. Methods in molecular biology (Clifton, N.J.). 731:237–245.

[16] Zhang G, McMullen RL, Kulcsar L (2016). Investigation of hair dye deposition, hair color loss, and hair damage during multiple oxidative dyeing and shampooing cycles. Journal of cosmetic science. 67: 1–11.

[17] Two AM, Del Rosso JQ (2014). Kallikrein 5-mediated inflammation in rosacea: clinically relevant correlations with acute and chronic manifestations in rosacea and how individual treatments may provide therapeutic benefit. The Journal of clinical and aesthetic dermatology. 7: 20–25.

[18] Zani MB, Sant'Ana AM, Tognato RC, Chagas JR, Puzer L (2022). Human Tissue Kallikreins-Related Peptidases Are Targets for the Treatment of Skin Desquamation Diseases. Frontiers in medicine. 8: 777619.

[19] Brattsand M, Stefansson K, Lundh C, Haasum Y, Egelrud T (2005). A proteolytic cascade of kallikreins in the stratum corneum. The Journal of investigative dermatology. 124:198–203.

[20] Powell AM, Sakuma-Oyama Y, Oyama N, Black MM (2005) Collagen XVII/BP180: a collagenous transmembrane protein and component of the dermoepidermal anchoring complex. Clinical and experimental dermatology. 30:682-687.

[21] Tanimura S, Tadokoro Y, Inomata K, Binh NT, Nishie W, Yamazaki S, Nakauchi H, Tanaka Y, McMillan JR, Sawamura D, Yancey K, Shimizu H, Nishimura EK (2011) Hair follicle stem cells provide functional niche for melanocyte stem cells. Cell stem cell. 8:177-187.

[22] Soma T, Tajima M, Kishimoto J (2005) Hair cycle-specific expression of versican in human hair follicles. Journal of dermatological science. 39:147-154.

[23] Watanabe M, Natsuga K, Nishie W, Kobayashi Y, Donati G, Suzuki S, Fujimura Y, Tsukiyama T, Ujie H, Shinkuma S, Nakamura H, Murajami M, Ozaki M, Nagayama M, Watt FM, Shimizu H (2017) Type XVII collagen coordinates proliferation in the interfollicular epidermis. eLife. 6:e26635.

[24] Gonzalez-Rico J, Quilez C, Lopez V, Muñoz-Barrutia A, Jorcano JL, Velasco D (2019) The role of versican in the skin ECM and its interaction with hyaluronic acid. Biomecanica. 27:35-49.

[25] Wu YJ, La Pierre DP, Wu J, Yee AJ, Yang BB (2005) The interactions of versican with its binding partners. Cell research. 15:483-494.