Revinage®

Description

A safe, stable, plant-based alternative to retinoids for the treatment of aging skin. **Revinage®** is an exclusive supercritical apolar extract of Picão Preto (*Bidens pilosa*) standardized in phytol.

Available in a palm-free version **(Revinage® WPO).**

Available in a RSPO version **(Revinage® MB).**

INCI

Revinage® and Revinage® MB: Bidens Pilosa Extract (and) Elaeis Guineensis (Palm) Oil (and) Gossypium Herbaceum (Cotton) Seed Oil (and) Linum Usitatissimum (Linseed) Seed Oil.

Revinage® WPO: Astrocaryum Murumuru Seed Butter (and) Bidens Pilosa Extract (and) Gossypium Herbaceum (Cotton) Seed Oil (and) Linum Usitatissimum (Linseed) Seed Oil.

Introduction

Retinoids are a family of compounds related to vitamin A and its derivatives that bind to specific receptors – stimulating pharmacological actions related to vision, reproduction, embryogenesis, regulation of inflammation, proliferation, growth and normal and neoplasia cell differentiation[1-3]. They were introduced to dermatology in the early 60s with the wide commercialization[2] of synthesized compounds. Retinoids are among the most prescribed dermatological therapeutic agents for the treatment of many common, yet clinically important skin diseases, such as acne, psoriasis, rosacea, hyperpigmentation and ichitiose, in addition to its benefits for the treatment of intrinsic and extrinsic aging[2,3].

Biologically the retinoids exert their effects by binding to nuclear receptors, known as RARs (Retinoic Acid Receptors) and RXRs (Retinoids X Receptors)[3]. After crossing the cell membrane, retinoids bind to some free proteins in the cytoplasm of the cell forming a compound that allows access to the interior of the cell nucleus. In the nucleus, the retinoids bind to RAR or RXR receptors forming a compound that has the ability to connect to specific regions of DNA, known as RARE (Retinoic Acid Responsive Element). Consequently, it starts the transcriptional activity related to the action of retinoids[2,3] including epidermal proliferation and differentiation, proliferation of fibroblasts as well as collagen, elastin and glycosaminoglycans production, essential components of the extracellular matrix. This capability makes them also applicable to the treatment and prevention of stretch marks[3].

The RAR receptor can also interact with other protein complexes of jun/fos type that stimulate interaction with the transcription factor AP-1 (activator protein-1) that, in turn, regulates the transcription of many other genes including metalloproteinases, growth factors (TGF-β), immune and inflammatory mediators[3,4]. The AP-1 is primarily induced by the exposure of the skin to ultraviolet radiation and, retinoids act as AP-1 antagonists, thereby preventing the transcription of the genes stimulated by this factor, being very effective in treating photoaging[3,4].

In addition, studies have shown that retinoids work on reduction of free radicals induced by inflammatory processes and therefore can act by accelerating the healing process of the skin, as well as contribute significantly to the reduction of pruritus, burning, dryness, appearance of wrinkles and hyperpigmentation associated with oxidative events[3-5]. However, some studies report a pro-oxidant action of retinoids and their derivatives in different cell types, including the stimulation of the natural antioxidant defense system, such as catalase and superoxide dismutase, because of this oxidative imbalance[6,7]. The disproportion, created by the formation of free radicals and/or the failure of the antioxidant system of the skin, generates lipid peroxidation, protein degradation and modification of genetic material leading to accelerated skin aging and the initiation of pathological processes such as inflammation[5].

In fact, oxidative damage of DNA in humans occurs more than 100 times a day[8] contributing to the degenerative effects of aging. As the nuclear DNA cannot be replaced, eukaryotic cells have developed different ways to fix it. Sirtuin 6 (SIRT6), known as the longevity molecule, is an enzyme which initiates DNA repair directly by stimulating one of the factors involved in the process. It also minimizes the production of reactive oxygen species by indirectly controlling the oxidative damage to DNA and other cellular structures[8]. In addition, SIRT6 stabilizes the telomeres, that consist of small random repetitions of DNA, associated with proteins at the ends of chromosomes. Oxidative stress, among other factors, also causes the progressive shortening of telomeres, which are consequently reflected in the loss of reproduction capacity of cells and cellular senescence[8].

Therefore, while the redox properties of the retinoids appear to vary in biological systems, they should not be ignored in a skin treatment using this therapeutic agent since the acceleration of aging and cellular senescence may compromise the long term results.

In addition to functioning on prevention and treatment of the clinical signs of aging, retinoids are widely used in the treatment of severe acne for reducing the production of sebum, normalizing the follicular keratinization, reducing colonization by *Propionibacterium acnes*, and reducing associated inflammation[9].

Retinoids Classification

The classification of retinoids is made based on the presence of aromatic groups being distributed across generations (Table 1)[2].

Table 1. Retinoids Classification

Generation	Some Examples
First (nonaromatic)	Retinol (all-trans-retinol, Vitamin A) Retinyl palmitate Retinaldehyde Tretinoin (all-trans-retinoic acid) Isotretinoin (13-cis- retinoic acid)
Second (monoaromatic)	Etretinate Acitretin
Third (poliaromatic)	Adaptalen Tazoroten
Forth (pyranones)	Selectinoide G Arotinoid

The newer generations of retinoids are targeted at specific RAR receptors such as Retinoic acid, which is the most active cell configuration of retinol and necessary for conversion into retinoic acid via an oxidative process, for greater efficacy[2]. Once converted, retinoic acid specifically activates the RAR receptor. This chemical configuration is known for an increase adverse effects when compared with retinol and its derivatives[10].

Problems of Classical Therapy with Retinoids

The side effects related to the use of retinoids are often associated with a skin irritation characterized by erythema, scaling, burning and itching. These effects are referred to as "retinoid dermatitis" and occurs in approximately 92% of all patients, a fact that limits therapy. Most patients report an unpleasant burning sensation when exposed to heat, in addition to photosensitivity. These patients should be instructed to avoid the sun or use sunscreen[2].

In certain cases, conjunctivitis irritation is also observed with the use of retinoids close to eyes. Additionally, due to its teratogenic and embryotoxic potential, pregnant or breastfeeding women should use topical retinoids with care, in spite of its very low (5-8%) absorption rate[11].

As these effects are dose dependent, patients should be advised to lower the dose and frequency of applications in cases of intense manifestations. This reduction in dosage will naturally reduce the effectiveness of therapy[2]. In addition, the common use of steroids to relieve the symptoms may have adverse effects when used for long periods[2].

The temporary, mild exacerbation of acne pustules in the first two weeks of use, hyperpigmentation and increased telangiectasia (dilated capillaries in the skin surface) are other adverse effects commonly identified[2,11]. Given these adversities for use with cosmetic products, alternative therapeutic agents to retinoids offer significant opportunities for the market[4,11].

Description of components

Picão Preto - *Bidens pilosa*

Bidens pilosa is an herbaceous plant, predominantly native to tropical areas of South America. It grows throughout Brazil, spreading widely through the Amazon rainforest, with the greatest concentration being in the South-Central agricultural region, where it is a flourishing species. It is also present on the West coast of the African continent and in Europe, mainly in the Iberian Peninsula. In Brazil, the plant grows throughout the year, but escalates in spring and summer, receiving the following common names: *picão, picão-preto, picão-campo, pico-pico, erva-picão, fura-capa, carrapicho-de-duas-pontas, goambu, coambi* etc.

Figure 1. Picão Preto (*Bidens pilosa*), flowers and fruits.

Picão-preto has been the subject of recent scientific research to support its applications in herbal and popular medicine. It is traditionally used in folk medicine for its anti-inflammatory, diuretic, anti-rheumatics and anti-diabetics properties[12]. Other studies show the functional use of *Bidens pilosa* as an anti-hypertensive[13], anti-ulcer[14] and antimalarial[15].

The phytochemical composition of *Bidens pilosa* is diversified, characterized primarily by the presence of flavonoids, terpenes, phenylpropanoids compounds, lipids and benzenoids[16]. One of the main chemical constituents described to *Bidens pilosa* is phytol, a diterpern present as part of the chlorophyll pigment. Phytol stands out because some studies show that when metabolized, it is oxidized to phytanic acid (also present in *Bidens pilosa*) that is able to bind to RXR receptors, activating them as retinoids and their derivatives[17,18] in very low concentrations such as 64 µM[17]. In addition, it also stimulates transcriptional activity of RAR[18], giving it "retinol-like" properties.

Due to its apolar characteristic, **Chemyunion** used the CO_2 supercritical extraction for phytol and its derivatives concentration in the creation and production of **Revinage®**, a plant-based alternative to retinoids in

treating aging skin. It operates using the same mechanisms, and demonstrates excellent performance without offering adverse effects or stability problems.

This extraction of supercritical fluids provides advantages over the conventional processes for obtaining active ingredients from a plant matrix, because it promotes the collection and separation of more purified compounds in higher concentrations, while reducing the presence of toxic compounds caused by thermal degradation of the active, and by not requiring application of organic solvents in the extraction process[19,20].

Benefits

Revinage® provides "retinol-like" behavior by binding to the RXR receptor and reducing by 20% the RAR expression while increasing the RXR expression by 119% resulting in effective anti-aging and anti-photoaging action:

- Regulates cell proliferation and differentiation as well as stimulates the production of extracellular matrix by ensuring greater skin firmness and wrinkles filling through:
    - 108% and 159% of stimulus in gene expression of EGFr and pro-collagen, respectively;
    - stimulation of 24% and 26% in the production of type I collagen and elastin, respectively;
    - stimulation in the production of collagen type IV;
    - 33% reduction of the production of MMP-1 in basal condition;
    - stimulation of 138% and 41% in the production of FGF-β and TGF- β, respectively;
- Offers antioxidant activity and senescence regulation due to:
    - stimulation of 247% and 47% in SOD and CAT, respectively;
    - 54% reduction in free radicals;
    - 30% reduction in MDA (marker of lipoperoxidation)
    - increase of 72% in SIRT6 gene expression;
- Provides a soothing effect:
    - 34% reduction in AP-1 activity;
    - reduction of 69%, 26% and 28% in the productions of COX-2, PGE2 and LTB4, respectively;
- Controls skin oiliness as it reduces DHT in 64%;
- Offers skin lightening activity when:
    - decrease in 72% the production of alpha-MSH;
    - reduce the production of melanin in 56%;
    - reduce in 37% the hyperpigmented regions in the face after 42 days of treatment
- Increases by 13% the elasticity of the facial skin of volunteers after 42 days of treatment;
- Increases by 25% the re-densification of the dermis in the face after 42 days of treatment;
- Reduces the roughness of the facial skin in 36% after 42 days of treatment;
- Reduces in 28% the deep wrinkles of the facial skin after 42 days of treatment;
- Controls the cutaneous barrier:

- Increase of 92.78% in the production of NMF after 2 hours of treatment;
- Increase of 55.85% and 12,25% in the production of ceramide-3 and cholesterol, respectively, after 4 hours of treatment;
- Reaches the deepest layers of the skin in 4 hours;
- Defends against photodegradation and oxidation.

Tests

Efficacy Test

1. *In silico* Evaluation

Using molecular modeling techniques, the simulation of the interaction between the RXR receptor and the phytanic acid was performed. Phytanic acid is the molecule produced through the transformation of the phytol present in **Revinage®** as it permeates the skin (figures 2 and 3).

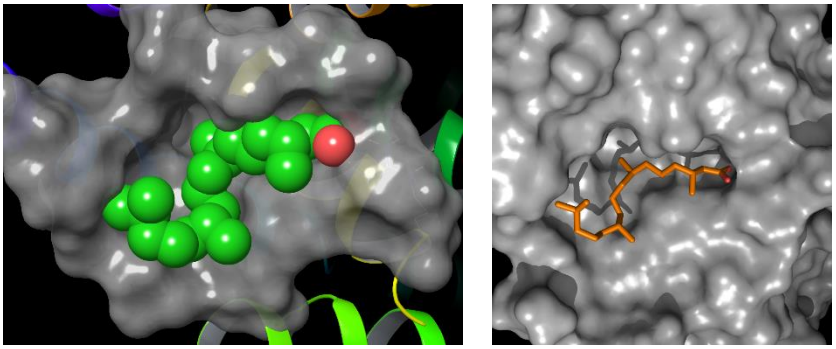


Figure 2. Phytanic acid (represented by green spheres on the left and orange sticks on the right) positioned on the active site of the RXR receptor (gray surface).

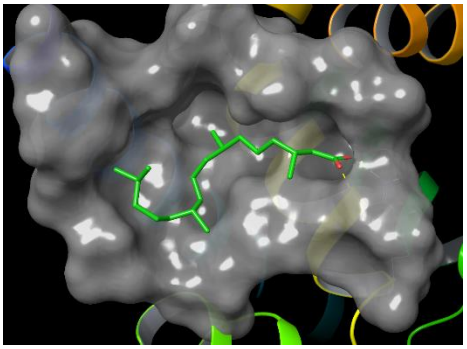


Figure 3. Phytanic acid (represented by green sticks) positioned on the active site of the RXR receptor (gray surface). Detail for the hydrogen bonds (dashed) between the oxygen atoms of the phytanic acid (in red) and the arginine R316.

In Figure 2, a good complementarity is shown between the RXR receptor cavity and phytanic acid. In Figure 3, it should be noted that the phytanic acid creates hydrogen bonding with the arginine R316 of the RXR receptor.

2. *In Vitro* Efficacy

Commercially obtained keratinocytes, fibroblasts and melanocytes were cultivated in a specific culture medium and maintained in a humid incubator at 37ºC containing CO_2 at 5%. For the tests, the cells were incubated with 0.15% of **Revinage®**, 30µM of retinoic acid and 0.15% of retinol for comparative purposes. The concentration of ingredients was based on pre-cytotoxicity tests by the MTT method.

After a 48-hour period, the supernatant and mRNA were collected for subsequent measurement of the proposed parameters. Variance Analysis (ANOVA) was used for statistical analysis except for gene expression assays. The Dunnet test was used when variance analysis detected significant differences between the groups with a 95% confidence interval.

2.1. RAR and RXR gene expression stimulation

After 48 hours of incubation, the total RNA of human fibroblasts was extracted using Trireactant ® solution and quantified using QuantityiT RNA™ Assay Kit (Byosystems applied) and the equipment Quibit ® Fluorometer. The relative quantity of mRNA was calculated as described in international studies[21].

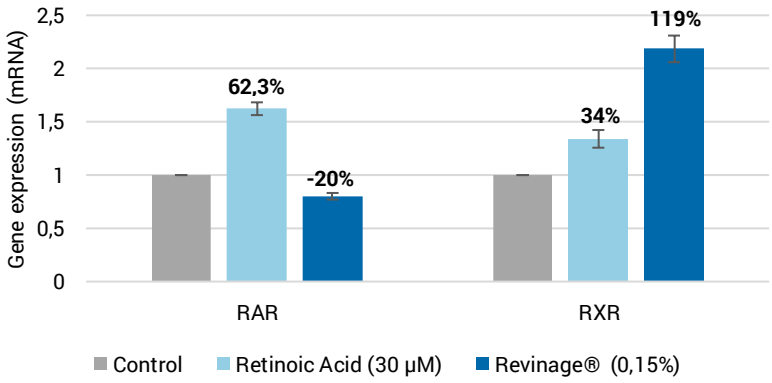


Figure 4. Relative gene expression of RAR and RXR in fibroblast cultures incubated with retinoic acid and Revinage®. The data represent the average ± deviation of 3 independent trials.

It was observed that **Revinage®** had been able to stimulate the expression of RXR in 119% and reduce the expression of RAR in 20%. It is also worth noting the suppression of RAR gene expression mediated by **Revinage®**, which can be related to a lower incidence of problems triggered by treatment with retinoids.

2.2. Cellular proliferation, differentiation and extracellular matrix (ECM) regulation

For the evaluation of the effects of **Revinage®**, retinoic acid and retinol on cellular proliferation, differentiation and extracellular matrix production, some studies were conducted on gene expression of the EGF receptor (EGFr) (epidermal growth factor receptor) and pro-collagen. In addition, the protein expression of the FGF-

β (fibroblast growth factor beta), TGF-β (tissue growth factor beta), metalloproteinase-1 (MMP-1), collagen and elastin were also performed.

After 48 hours of incubation with the actives, the total RNA of human fibroblasts was extracted using Trireactant ® solution and quantified using QuantityiT RNA™ Assay Kit (Byosystems applied) and the equipment Quibit ® Fluorometer. The relative quantity of mRNA was calculated as described in international studies[21].

The protein quantification of TGF-β, MMP-1, collagen and elastin was performed in the supernatant of culture of human fibroblasts through biochemistry detection using a commercially available kit (BD Biosciences, USA and Biocolor, USA).

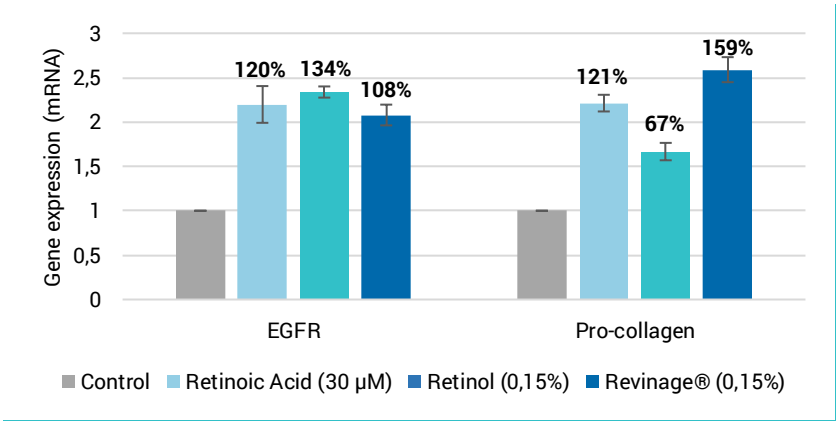


Figure 5. Relative gene expression of EGF receptor and pro-collagen in fibroblast cultures incubated with retinoic acid, retinol and **Revinage®**. The data represent the average ±deviation of 3 independent trials.

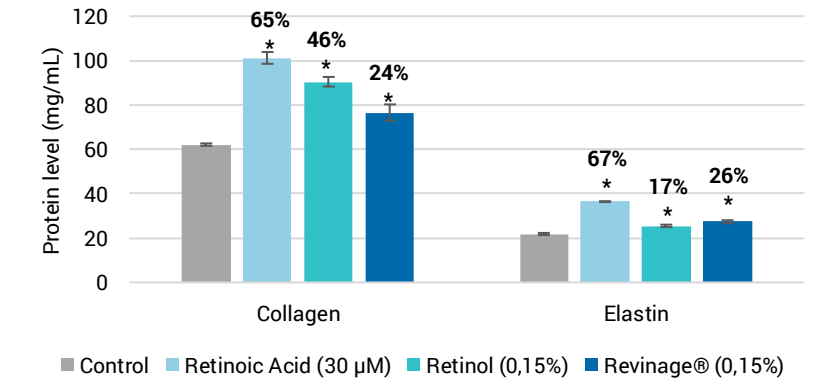


Figure 6. Quantification of type I collagen and elastin in fibroblast cultures incubated with retinoic acid, retinol and **Revinage®**. The data represent the average ± deviation of 3 independent trials. * p< 0.05 relative to the control.

EGF receptor, after activation, controlled both proliferation and cellular differentiation, in addition to other important cellular functions such as apoptosis, inflammation and imunity[22]. **Revinage®** was able to increase the gene expression of EGFr similarly to retinoic acid and retinol, as well as stimulating the expression of pro-collagen (Figure 5). Our results showed that both **Revinage®**, retinoic acid and retinol were able to increase the synthesis of

collagen and elastin (Figure 6), in addition to reducing the production of metalloproteinase-1 (MMP-1), main collagenase of the skin (Figure 7).

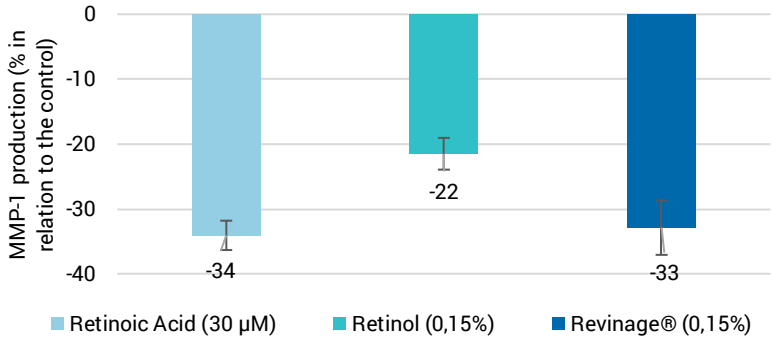


Figure 7. Quantification of MMP-1 in fibroblast cultures incubated with retinoic acid, retinol and **Revinage®** with respect to the control. The data represent the average ± deviation of 3 independent trials. All products presented p< 0.05 relative to the control.

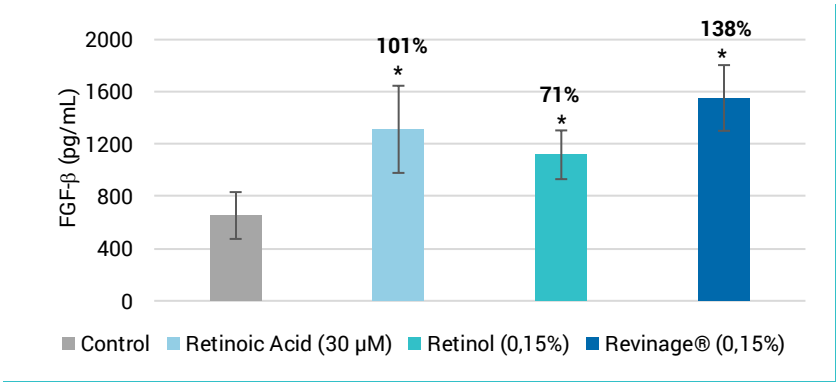


Figure 8. Quantification of FGF-β in fibroblast cultures incubated with retinoic acid, retinol and **Revinage®**. The data represent the average ± deviation of 3 independent trials. * p< 0.05 relative to the control.

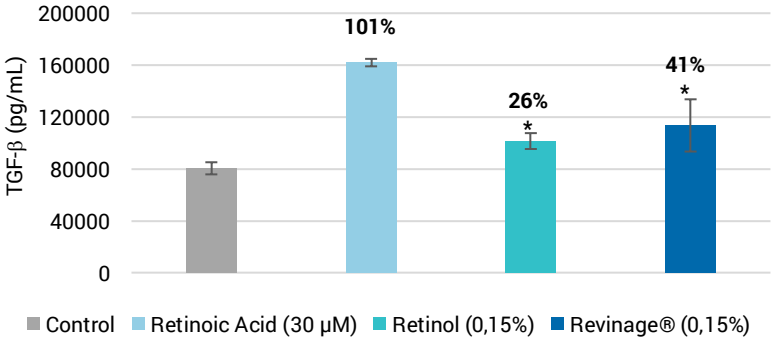


Figure 9. Quantification of TGF-β in fibroblast cultures incubated with retinoic acid, retinol and **Revinage®**. The data represent the average ± deviation of 3 independent trials. * p< 0.05 relative to the control.

This effect was mainly regulated by stimulation of FGF-β (Figure 8) and TGF-β (Figure 9). FGF-β and TGF-β, known growth factors, present a wide spectrum of activity including the increased synthesis of extracellular matrix, in addition to contributing to the proliferation of fibroblasts and inhibition of MMP-1[22].

2.3. Antioxidant activity and senescence regulation

A measurement of the effects of **Revinage®**, retinoic acid and retinol on the activity of SOD (superoxide dismutase) and CAT (catalase) was made of a culture cell lysate of human fibroblasts via enzymatic reactions using commercially available kits (Cayman Chemical, USA). As positive control was used the alpha-tocopherol to 0.15%, known antioxidant substance that stimulates enzyme activities of SOD and CAT.

According to the results (Figure 10 and 11), it was observed that **Revinage®** acts by stimulating by 247% and 47% the activity of natural skin antioxidant enzymes, SOD and CAT, respectively. SOD and CAT are the main forms of protection against the production of free radicals in the skin, being then able to prevent structural damage to biological oxidation-prone structures like lipids.

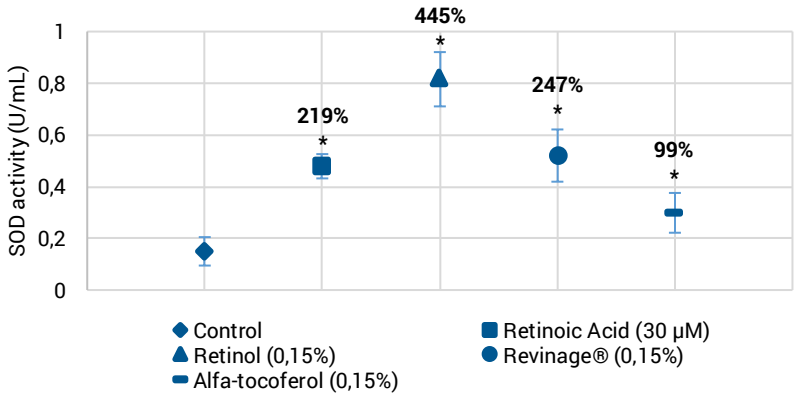


Figure 10. Quantification of SOD activity in fibroblast cultures incubated with retinoic acid, retinol, alfa-tocopherol and **Revinage®**. The data represent the average ± deviation of 3 independent trials and the percentages are related to the control. * p< 0.05 relative to the control.

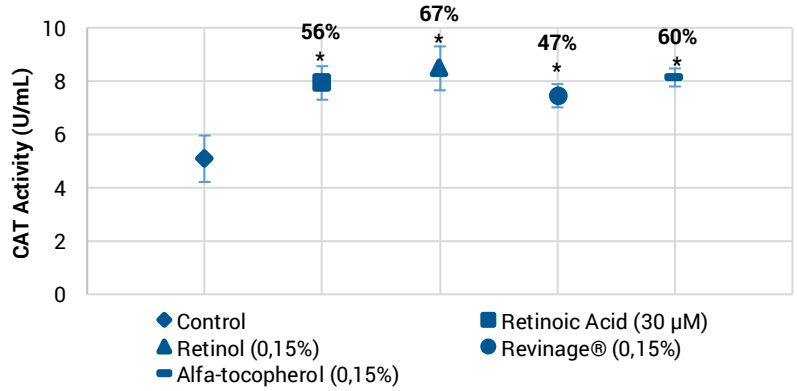


Figure 11. Quantification of CAT activity in fibroblast cultures incubated with retinoic acid, retinol, alfa-tocopherol and **Revinage®**. The data represent the average ± deviation of 3 independent trials and the percentages are related to the control. * p< 0.05 relative to the control.

However, due to the pro-oxidant effect report of retinoids stimulating the production of SOD and CAT, the production of free radicals stimulated by **Revinage®** was evaluated. It was performed by chemiluminescence kit (available commercially) in a mouse fibroblasts culture after a 30 minutes incubation period to ensure that the mechanism of activation of antioxidant enzymes was not caused by unbalanced oxidation in the treatment process.

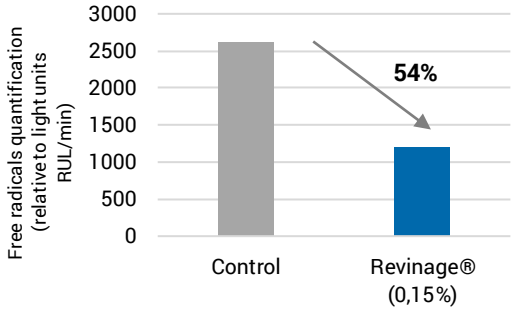


Figure 12. Quantification of the production of reactive oxygen species inhibited by **Revinage®**.

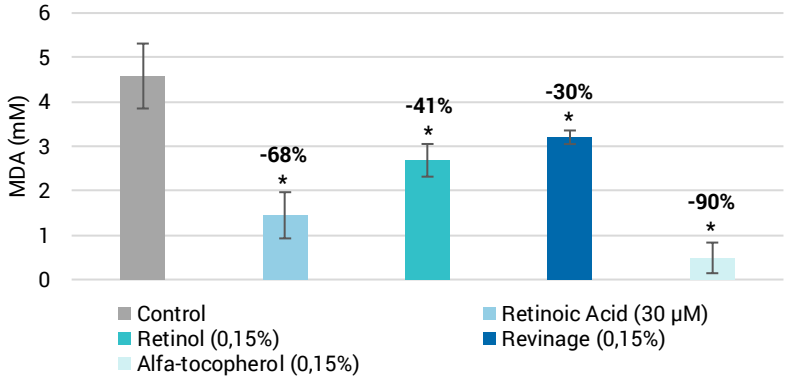


Figure 13. Quantification of malonaldehyde (MDA) in keratinocytes cultures incubated with retinoic acid, retinol, alfa-tocopherol and **Revinage®**. The data represent the average ± deviation of 3 independent trials and the percentages are related to the control. * p< 0.01 relative to the control.

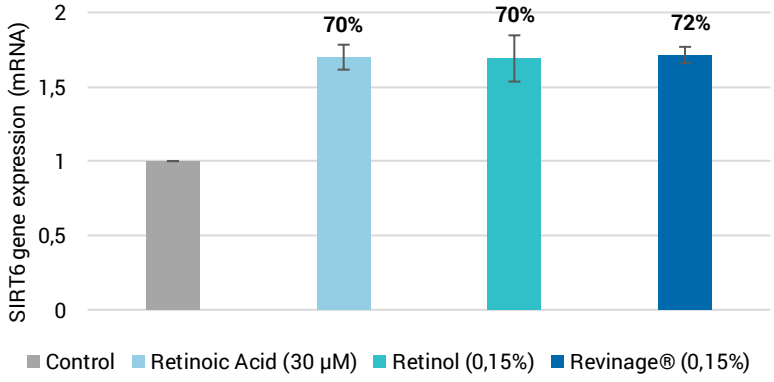


Figure 14. Relative gene expression of situin 6 (SIRT6) in fibroblast cultures incubated with retinoic acid, retinol and **Revinage®**. The data represent the average ±deviation of 3 independent trials.

The protective effect of skin structures can be seen in Figure 13, where **Revinage®** reduced by 30% the levels of MDA, a marker of lipoperoxidation. In addition, **Revinage®** stimulated by 72% the gene expression of SIRT6 that stimulates the repair of oxidative damage to DNA and stabilizes the telomeres, thus assisting and securing the proliferative capacity of cells while reducing the cellular senescence.

2.4. Soothing Activity

To evaluate the effect of **Revinage®**, retinoic acid and retinol in the inflammatory cascade stimulated by AP-1, studies of the activity of the transcription factor AP-1 in fibroblasts cell lysate were performed. Inflammatory mediators such as COX-2 (Ciclo-oxigenase-2), LTB4 (Leukotriene B4) and PGE2 (Prostaglandin E2) were measured in culture supernatant of keratinocytes after 48 hours of incubation. The measurements were made using immunoassays with commercially available kits (Cayman Chemical, USA; Millipore, USA; BD Biosciences, USA; IBL America, USA).

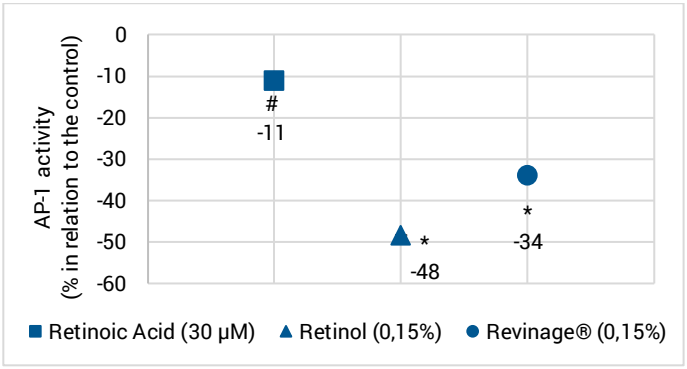


Figure 15. Quantification of AP-1 activity in fibroblast cultures incubated with retinoic acid, retinol and **Revinage®**. The data represent the average ± deviation of 3 independent trials and the percentages are related to the control. * p< 0.01 and # p<0,05 relative to the control.

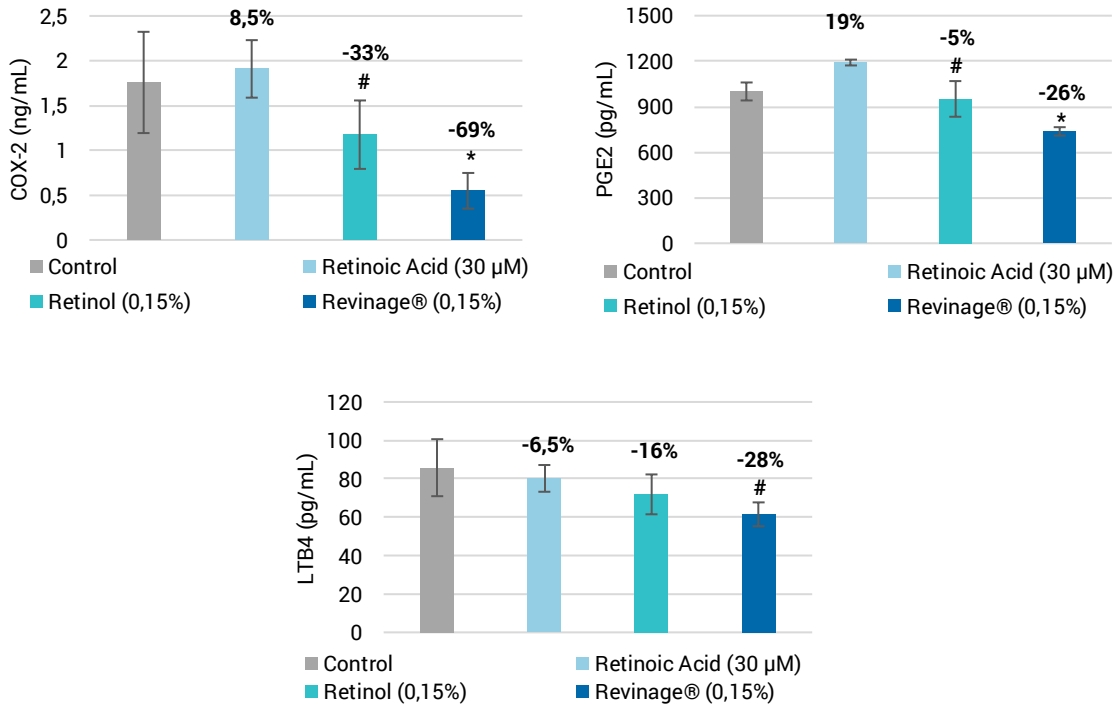


Figure 16. Quantification of COX-2, PGE2 and LTB4 in keratinocytes cultures incubated with retinoic acid, retinol and **Revinage®**. The data represent the average ± deviation of 3 independent trials and the percentages are related to the control. * p< 0.01 and # p<0,05 relative to the control.

It was observed that **Revinage®** inhibits AP-1 by 34% offering a similar result to retinol, while retinoic acid was able to inhibit only 10% of AP-1 activity (Figure 15). The AP-1 sequence is found in the promoter regions of numerous genes, including metalloproteinases of matrix, growth factors (TGF-β) and inflammatory mediators. Thus, AP-1 plays an important role in induction of AP-1's immune responses and antagonistic substances (inhibitors) can block their functionality and inhibit the synthesis of components of the inflammatory response[4], which are therefore related to the major adverse effects of retinoic acid[10].

Revinage® has demonstrated significant improvement of the major inflammatory mediators, COX-2, PGE2 and LTB4, while the retinoic acid has not demonstrated the ability to stimulate even the production of COX-2 and PGE2 (Figure 16). Even in comparison to retinol, **Revinage®** was observed to demonstrate a superior reduction of inflammatory mediators**,** which corroborates its small effect on the inflammatory cascade. These results demonstrate that **Revinage®** does not stimulate inflammatory pathways reported for the retinoids, presenting, therefore, greater safety for use in the treatment of the skin.

2.5. Oilness Regulation Activity

To evaluate the effect of **Revinage®** in the control of the production of sebum, the supernatant from human keratinocytes cell culture was evaluated for the production of DHT (dihydrotestosterone) using commercially available immunoassay (RayBiotech, USA). For comparative purposes, 0.05% of Sebonormine and 0.05% of Saw Palmetto extract (dry), known inhibitors of DHT production were used in the study.

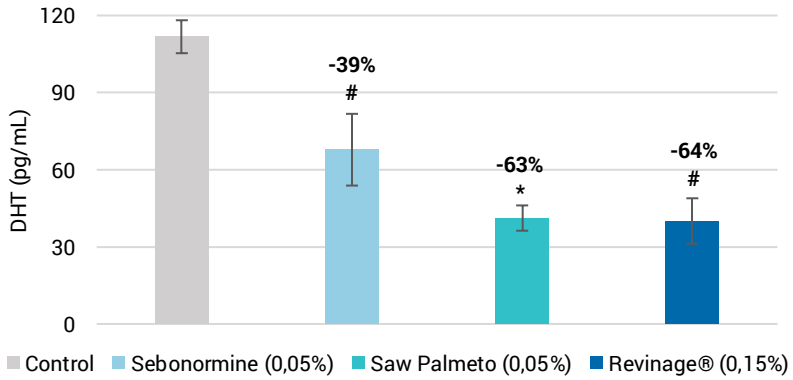


Figure 17. Quantification of DHT in keratinocytes cultures incubated with sebonormine, saw palmettol and **Revinage®**. The data represent the average ± deviation of 3 independent trials and the percentages are related to the control. * p< 0.01 and # p<0,05 relative to the control.

Revinage® in comparison with the control group, promoted a reduction in the production of DHT (64% reduction) demonstrating its *in vitro* effects to control the oiliness of the skin. **Revinage®** also showed greater efficacy in comparison with the active agents of reference, which promoted the reduction of 39% and 63%, respectively.

2.6. Whitening Effect

To evaluate the whitening effect of **Revinage®**, melanin content was measured in cultures of human melanocyte incubated for 48 hours. The cellular lysate was analyzed and compared using a standard melanin (Sigma-Aldrich, Cat. M0418) through the reading of absorbance (at 405 nm). For the purposes of comparing the whitening effectiveness, alpha-arbutin was used at 0.005% and kojic acid at 0.05%. The results are reported in Figure 18a.

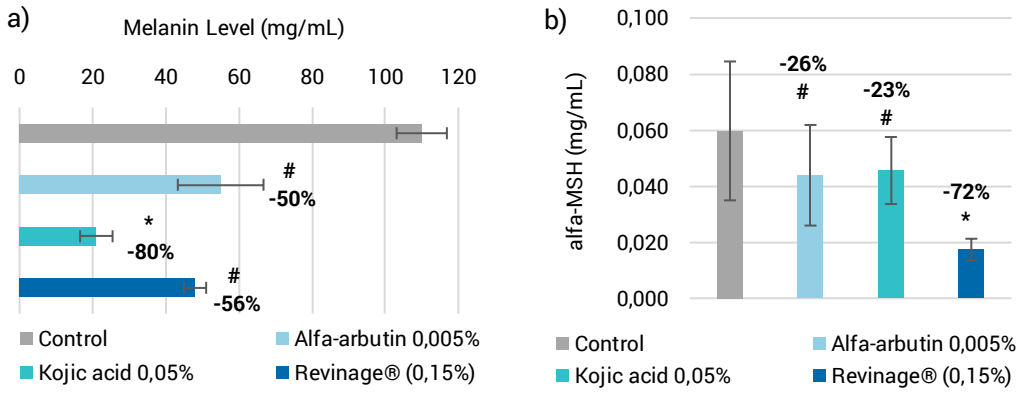


Figure 18. Quantification of melanin (a) and alfa-MSH (b) in melanocytes and keratinocytes cultures, respectively, incubated with kojic acid, alfa-arbutin and **Revinage®**. The data represent the average ± deviation of 3 independent trials and the percentages are related to the control. * p< 0.01 and # p<0,05 relative to the control.

The product provided a significant (56%) reduction of pigment in comparison with the control group. Arbutin and kojic acid also promoted reductions in the production of melanin, reducing approximately 50% and 80%, respectively, compared with cells that did not received the product. To determine the action mechanism of by which **Revinage®** provides its whitening effect, the production of alpha-MSH in the supernatant of human keratinocytes culture was evaluated using commercially available immunoassays (Pharmaceuticals, USA) (Figure 18b). The hormone αalfa-MSH plays an important role in the process of human skin pigmentation, because it binds to the melanocortin-1 receptor (MC1R) in melanocytes resulting in increased production of eumelanin.

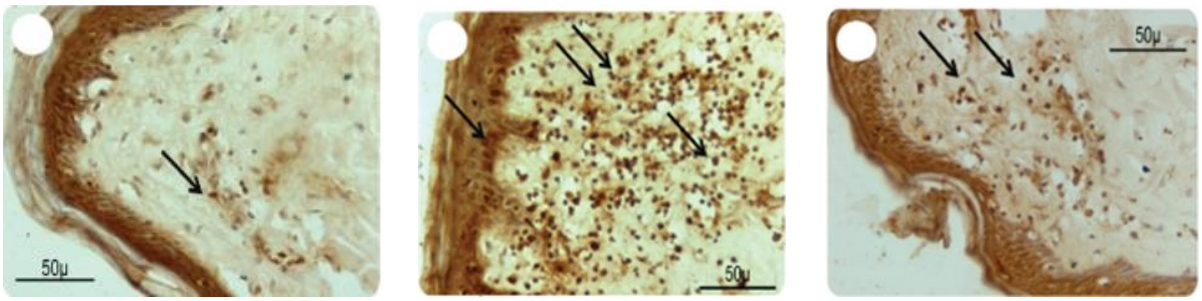
3. *Ex Vivo* Efficacy

3.1. RAR and RXR protein expression

Human skin fragments were incubated with 0.5% **Revinage®** for 48 hours. 0.5% Retinoic acid was used as positive control. After incubation, the samples were fixed and cryo-protected for future use of serials cuts.

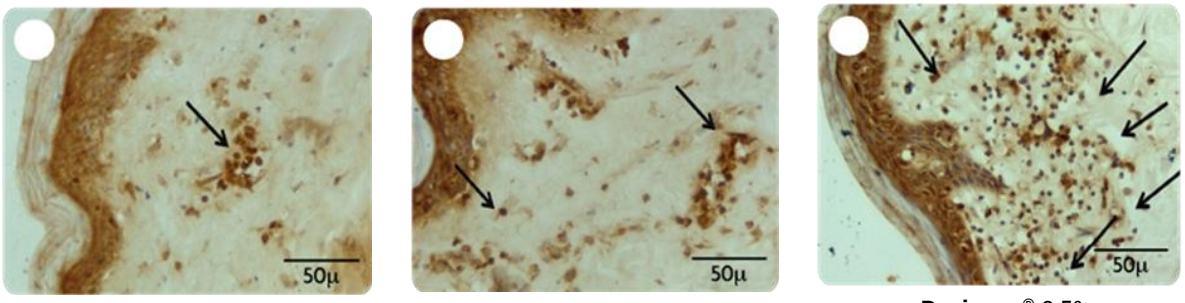
For the immunohistochemistry of RAR and RXR, histological sections were submitted to standard protocol as indicated by manufacturer of Histostain-SP Kit (ZymedLaboratories). Then the primary antibodies anti-RXR and anti-RAR were prepared and incubated overnight. Incubation with secondary antibodies and the results of the reaction followed the guidelines of the manufacturer.

The results confirm the in vitro studies that demonstrated the gene expression of both receivers. Again, it was clear that **Revinage®** is effective in stimulating the RXRs (Figure 20), whereas retinoic acid stimulates the subtype RAR (Figure 19).



Control | Retinoic Acid 30 µM | **Revinage®** 0,5%

Figure 19. Effect of the actives on the immunohistochemistry expression of RAR receptor in skin fragments: untreated fragment (control), Retinoic acid and **Revinage®**. Increase in 400x.



Control | Retinoic Acid 30 µM | **Revinage®** 0,5%

Figure 20. Effect of the actives on the immunohistochemistry expression of RXR receptor in skin fragments: untreated fragment (control), Retinoic acid and **Revinage®**. Increase in 400x.

3.2. Extracellular Matrix (ECM) protein expression

To determine the influence of the treatment on the production of type I and type IV collagen, the human skin fragments were incubated with 2.0% **Revinage®** or 2.0% retinol for 24 hours. After incubation, the samples were fixed and cryo-protected for future use of serials cuts. Histological sections were subjected to standard protocol including washing, overnight incubation with primary antibodies (Sigma, USA), and subsequent incubation with secondary antibody (Invitrogen, A11001). The nuclei were marked with DAPI (4 '-6-Diamidino-2-Fenilindol), a known intercalant of DNA. The analysis was conducted through bright field and fluorescence microscopy in a Leica-DM1000 with the aid of the LAS Software (Leica application suite).

The assessment of the effects of **Revinage®** in the production of collagen type I and IV can be easily identified by the intense green fluorescence in Figure 21, being similar to that found for the retinol in the same concentration. These data corroborate the effect of dermal re-densification indicating greater activity in the tissue regeneration process, thus preventing skin aging and wrinkle formation.

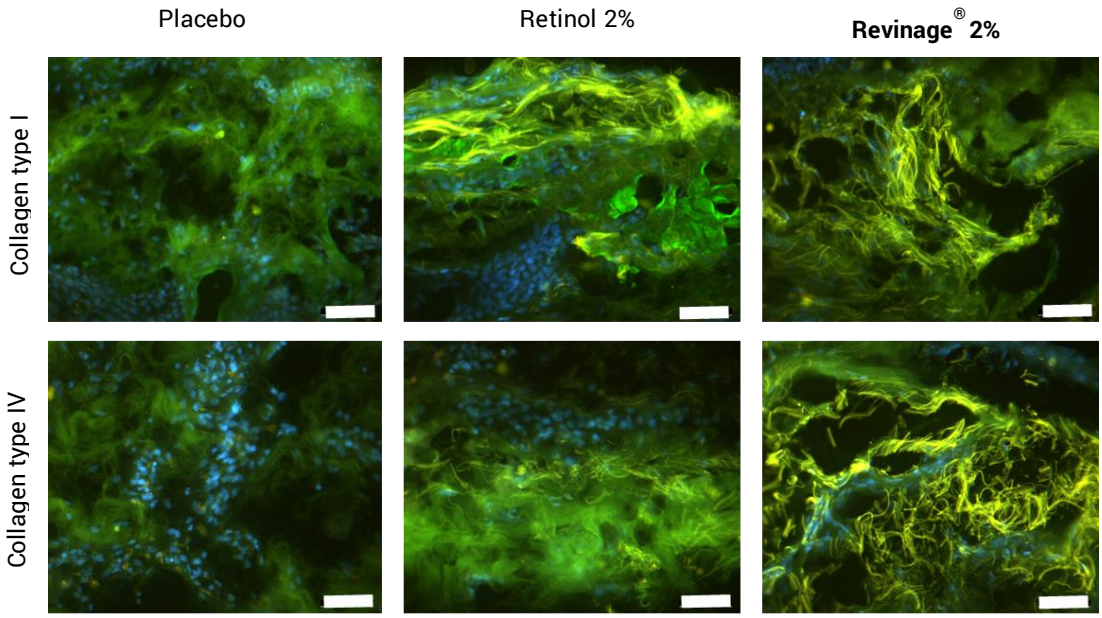


Figure 21. Effect of **Revinage®** and retinol palmitate in the synthesis of collagen type I and IV in skin fragments marked with antibodies after 24h incubation by controlling untreated control. Size = 50 µm.

Furthermore, after 24 hours of incubation, some skin fragments were subjected to UV radiation (3J/cm^2) using a solar simulator (SOL-500; Hönle). After photostimulation, the fragments were again treated with 2.0% **Revinage®** or 2.0% retinol for 24 hours and, subsequently macerated for detection of MMP-1 through commercially available ELISA kits (R&D systems, Minneapolis, USA). Variance Analysis (ANOVA) was used for statistical analysis and the Dunnet test was used with 95% confidence range.

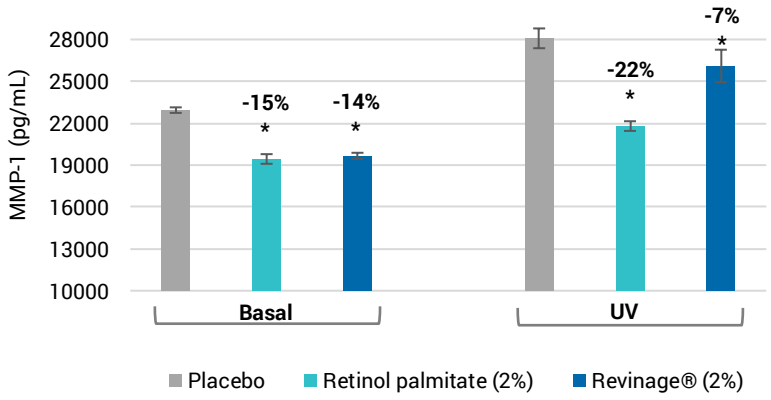


Figure 22. Effect of **Revinage®** and retinol palmitate in the production of MMP-1 in skin fragments treated or not with UV radiation (3J/cm^2). * p < 0.01 and # p < 0, 05 relative to the control.

It is observed that **Revinage®** was able to decrease the production of MMP-1 in basal condition in a manner equivalent to retinol (Figure 22). Even when exposed to UV radiation, **Revinage®** has significantly modulated the production of MMP-1, proving to be a great ally against photoaging (Figure 22).

3.3. Extracellular Matrix (ECM) protein expression *versus* Backuchiol

To ensure the firmness and elasticity of the skin, some extracellular matrix proteins are essential to maintain the integrity of the skin tissue, e.g., type 1 collagen (COL1) is mainly responsible for the mechanical properties of the skin and its reduction in synthesis is a characteristic of endogenous skin. Additionally, COL1 fibrils do not form or stabilize without fibronectin, an extracellular matrix glycoprotein essential in cell adhesion, growth, migration, and differentiation [24].

Type IV collagen (COL4) its main function is to maintain the integrity of the dermo-epidermal junction to ensure its functionality and the nutrition of the basal layer epidermal cells. The reduction of type IV collagen (COL4) compromises the mechanical stability of the skin tissue and negatively interferes with tissue regeneration since mechanical stability is required for the synthesis of new molecules45. As the skin ages, COL4 is also reduced[26].

Another important protein that comprises the ECM is elastin. Its presence is essential to the maintenance of the skin elastic properties and has the main function to store energy and promote passive recoil of composite structures known as elastic fibers, a highly insoluble and ordered protein that provides skin elasticity[27].

Based on the information listed above, a study was carried out on human skin fragments to evaluate the potential of **Revinage®** against Backuchiol in essential markers for skin firmness and elasticity.

3.3.1. Collagen Type I production (Revinage® *versus* Backuchiol)

Human skin fragments from blepharoplasty of five healthy patients aged 45-60 years were treated for 72 hours with 10 mg/cm^2 of formulas containing 0,5% Backuchiol or 1% Backuchiol or 1% **Revinage®,** in addition to a placebo formulation. 10 µm sections were collected incubated with primary Anti-Collagen 1 (Abcam; ab90395) antibodies followed by incubation with secondary antibody (Invitrogen A11001) and DAPI counterstained (4'-6-Diamidino-2-phenylindole, marker of DNA).

The fluorescence intensity emitted by specific antibodies used in the treatment of the sections was analyzed with the Fluorescence Microscope (Leica-DM1000 / LAS-Leica Application Suite). The images obtained were treated with Software ImageJ® to quantify the pixels generated by the target protein.

Figure 23 demonstrates collagen-I synthesis in different samples compared to the placebo formulation.

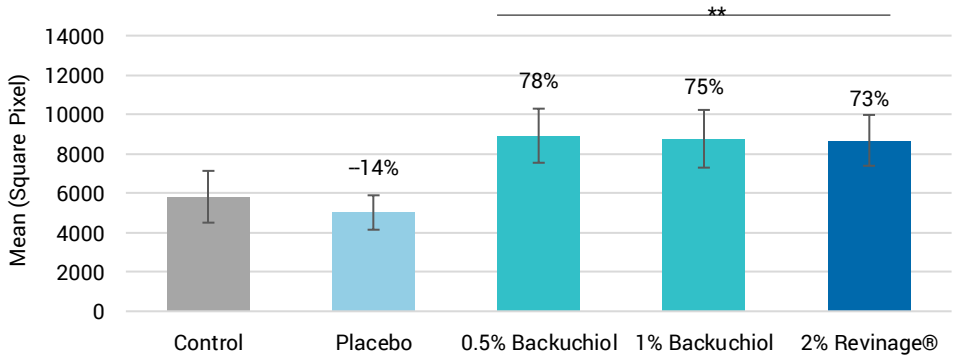


Figure 23. Effect of **Revinage®** and Backuchiol in the production of collagen type I in skin fragments.
**p < 0.05 relative to the control.

According to the results, it is possible to observe that both Bakuchiol and **Revinage®**, in their concentration of use, significantly increase the production of type I collagen, however, there is no significant difference between the results obtained for the Bakuchiol and **Revinage®** groups ($p>0.05$). Furthermore, it is interesting to note that regardless of the concentration of Bakuchiol, the same performance was achieved in collagen production.

3.3.2. Collagen type IV, elastin and fibronectin production (Revinage® *versus* Backuchiol)

Human skin fragments from blepharoplasty of five healthy patients aged 45-60 years were treated for 72 hours with 10 mg/cm^2 of formulas containing 1% Backuchiol or 1% **Revinage®,** in addition to a placebo formulation**.** 10 µm sections were collected incubated with primary Anti-Collagen 4 (Santa Cruz Biotech, sc-52317) or Antielastin (Santa Cruz Biotech, sc25736) or Anti-Fibronectin (Santa Cruz Biotech; sc 69681), antibodies followed by incubation with specific secondary antibody and DAPI counterstained (4′-6-Diamidino-2-phenylindole, marker of DNA).

The fluorescence intensity emitted by specific antibodies used in the treatment of the sections was analyzed with the Fluorescence Microscope (Leica-DM1000 / LAS-Leica Application Suite). The images obtained were treated with Software ImageJ® to quantify the pixels generated by the target protein.

Figures 24, 25 and 26 demonstrates the proteins synthesis of the markers synthesis in different samples compared to the placebo formulation.

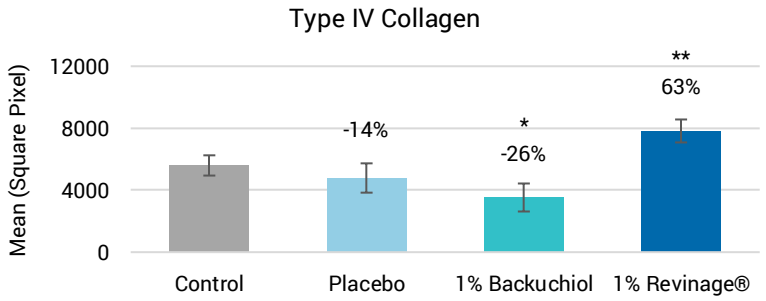


Figure 24. Effect of **Revinage®** and Backuchiol in the production of collagen type IV in skin fragments. **p < 0.01 and *p<0.05 relative to the control.

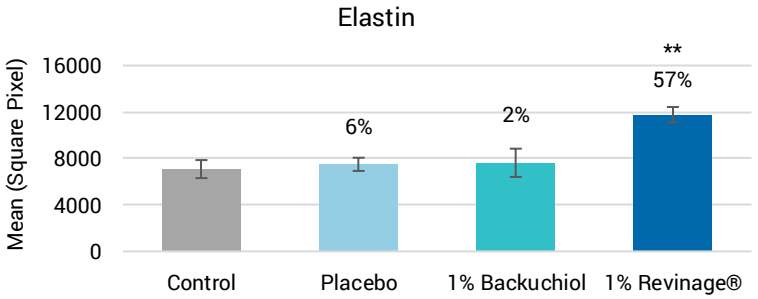


Figure 25. Effect of **Revinage®** and Backuchiol in the production of elastin in skin fragments. **p < 0.01 and *p<0.05 relative to the control.

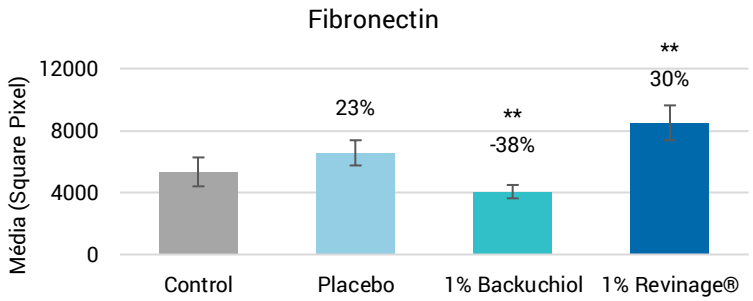


Figure 26. Effect of **Revinage**® and Backuchiol in the production of fibronectin in skin fragments. **p < 0.01 and *p<0.05 relative to the control.

It is noted that although Backuchiol and **Revinage**® have demonstrated similar efficacy in the production of type 1 collagen (Figure 23), the same cannot be seen for the other investigated biomarkers according to the evidence raised in Figures 24, 25 and 26. Thus, **Revinage**® demonstrated to have broad action in improving the quality of the skin tissue, with a significant increase of ECM essential proteins, which is expected in a retinol-like ingredient.

4. *In Vivo* Efficacy

The clinical efficacy assessment of **Revinage**® was conducted in 40 healthy female volunteers aged 35-55 years with phototypes type II, III and IV (Fitzpatrick). The volunteers were instructed to apply the two formulations containing 2% **Revinage**® and 0.3% retinol palmitate, in the region of the forearms and the face (being a formulation in each forearm and hemi-face), twice a day, for a period of 42 days.

Instrumental measurements and documentation were conducted before the start of treatment and after 14, 18 and 42 days of treatment and included:

- Instrumental evaluation of skin elasticity using CUTOMETER SEM 575;
- Instrumental evaluation of densification of the skin using the DUB®- USB ultrasound;
- Instrumental evaluation of UV hyperpigmentation using VISIA®;
- Instrumental evaluation of roughness of skin surface and wrinkles using PRIMOS®;
- Standardized photographic record by PRIMOS® and OMNIA®.

Revinage® was also applied as a lipstick using a 1% formulation for assessing its benefits in hydration and restructuring of the labial semi-mucous. This evaluation was made by Raman Spectroscopy.

Furthermore, the cutaneous permeation of **Revinage**® was evaluated through Raman Spectroscopy using phytol (Sigma, W502200) as a standard in two volunteers who applied the formulation containing 3% **Revinage**® and its placebo a single-time on the forearm. The spectrum of phytol was acquired after 30 minutes, 1h, 2h, 4h, 18h and 24h of the application and the result expressed in % average of phytol found between 1-60 µm deep into the skin.

4.1. Instrumental evaluation of skin elasticity using CUTOMETER SEM 575

For the evaluation of skin elasticity, the parameter R2 was used, and calculated by the ratio between the maximum amplitude (Ua) and the resiliency of the skin (gross elasticity – Uf)[28]. This evaluation was performed only on the facial skin and the basal values compared to the values obtained after the days of treatment.

The formulation containing **Revinage®** provided a satisfactory increase in R2 parameter values, which indicates improvement in skin elasticity. It was not possible to observe statistical differences between the results obtained with the formulations containing **Revinage®** and retinol palmitate.

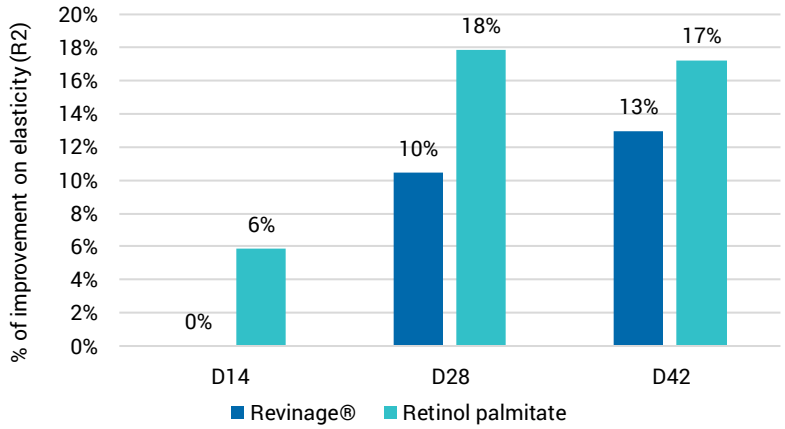


Figure 27. Effect of **Revinage®** and retinol palmitate in the elasticity of the facial skin.

4.2. Instrumental evaluation of densification of the skin using DUB®-USB ultrasound

Using the ultrasound technique it is possible to evaluate the density of the skin, through the difference of echogenicity of the evaluated regions. The equipment uses software that provides results in ultrasound units. Increase in ultrasound units indicates improvement in the density of the skin, which is related to the state of the collagen and elastin fibers of the dermis[29]. For this measurement, both regions of the face and forearm were evaluated.

The formulation containing **Revinage®** provided a noticeable increase in the number of ultrasound units, indicating improvement in cutaneous density, which may be related to an improvement in the collagen and elastin fibers of the dermis. It was not possible to observe statistical differences between the results obtained with the formulations containing **Revinage®** and retinol palmitate.

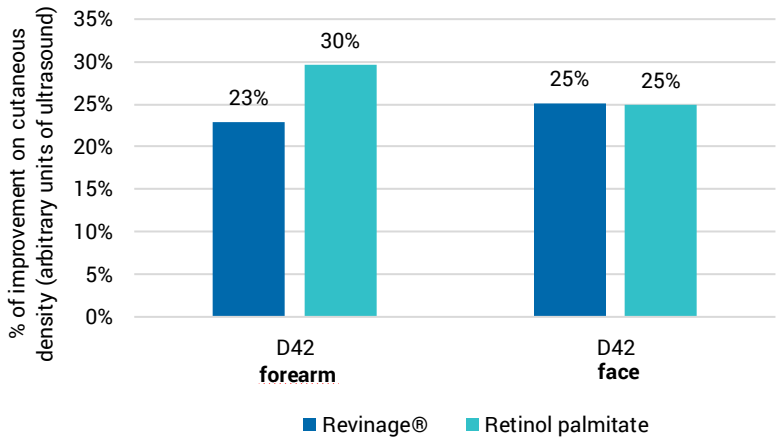


Figure 28. Effect of **Revinage®** and retinol palmitate in the re-densification of the facial and forearm skin.

4.3. Instrumental evaluation of UV hyperpigmentation using VISIA®

UV hyperpigmentation is usually invisible under normal lighting conditions. Selective absorption of UV light by epidermal melanin increases its display and detection by VISIA® equipment. This evaluation was performed only on the skin of the face and the basal values compared to the values obtained after the days of treatment.

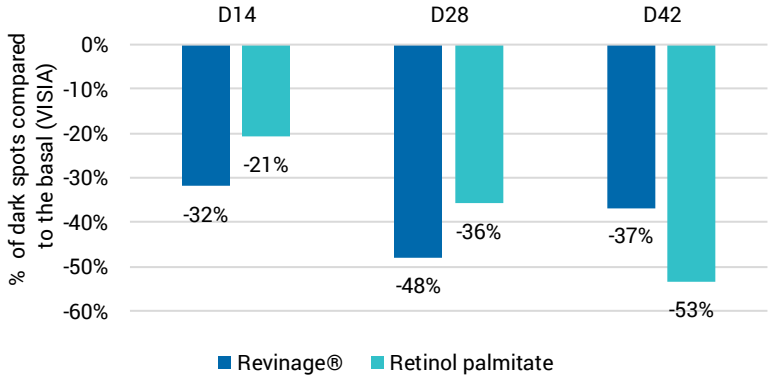


Figure 29. Effect of **Revinage®** and retinol palmitate in the UV hyperpigmentation of the facial skin.

The formulation containing **Revinage®** sharply reduced the hyperpigmentation over time compared to the basal control and there was no statistical difference in relation to the treatment with retinol palmitate.

4.4. Instrumental evaluation of roughness of skin surface and wrinkles using PRIMOS®

The evaluation of cutaneous micro-relief and wrinkles was performed through the study of the Sa and Smax parameters, obtained using PRIMOS®, a three-dimensional analytical system capable of performing in vivo measurements of the morphological structure of the human skin[30].

The Sa parameter is the arithmetic mean of quantitative measurements representing the topographical profile of the skin surface. The decrease in the Sa values may indicate an improvement in the roughness and texture of the skin[30]. The Smax parameter is the distance between the highest and the deepest point of the skin surface, which represents the maximum depth of the wrinkles. The decrease in Smax values can indicate an improvement in the wrinkles of the skin[30]. For this measurement, the regions of the face and forearm were evaluated.

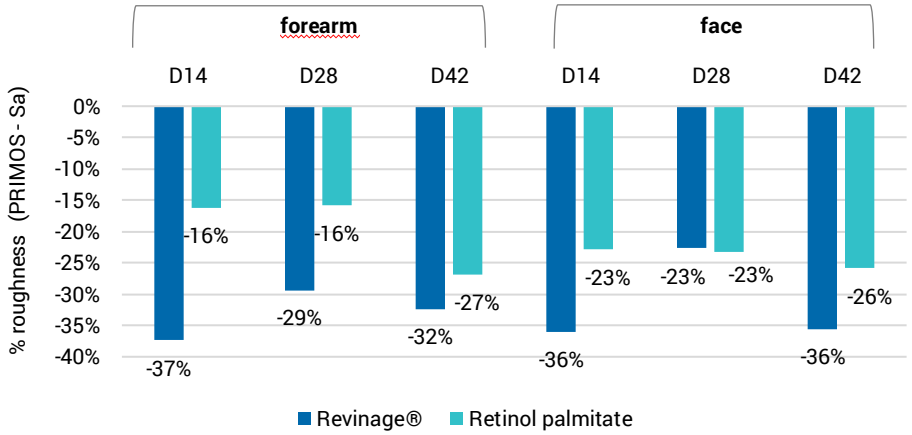


Figure 30. Effect of **Revinage®** and retinol palmitate in the skin roughness.

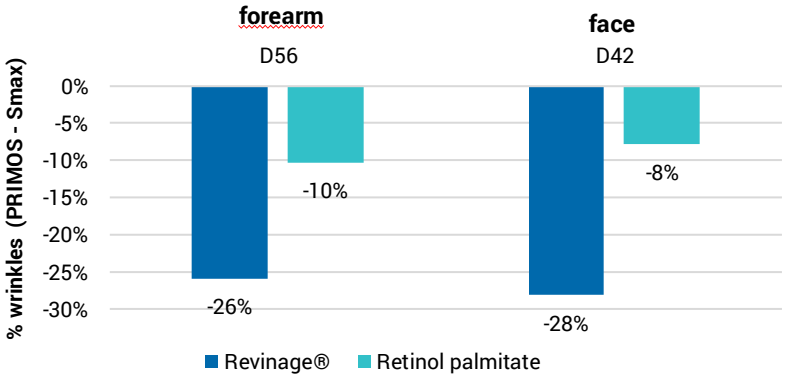


Figure 31. Effect of **Revinage®** and retinol palmitate in the skin wrinkles.

The formulation containing **Revinage®** provided a decrease in the values of the Sa (Figure 30) and Smax (Figure 31) parameter, indicating improvement in the roughness and texture and in the deep wrinkles of the skin, respectively. It was not possible to observe statistical differences between the results obtained with the formulations containing **Revinage®** and retinol palmitate.

As an example of the photographic documentation obtained and generated by PRIMOS®, by using the values from Smax (Figure 32) and, a decrease in the deeper wrinkles, even after 28 days of treatment with **Revinage®**, can be clearly observed.

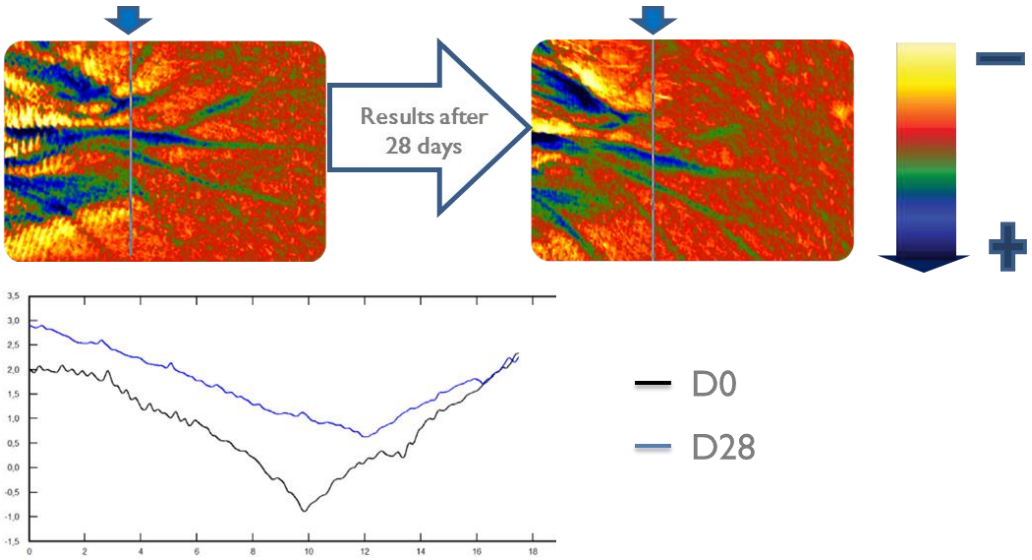


Figure 32. Revinage® effect on deep wrinkles of the facial skin (periorbital region) after 28 days of continuous treatment. Wrinkle depth indicated by colored arrow on the right (+ signal indicates the deepest measurements). The graph shows values of Smax obtained in the regions indicated by arrows in the photographs. On the chart the black line indicates Smax in D0 while blue line indicates Smax retrieved from D28.

4.5. Standardized photographic record by OMNIA®

OMNIA® (Canfield, USA) allows the acquisition of highdefinition images in a standardized and reproducible form, being ideal for photographic documentation of clinical studies.

We can see that the treatment with 2% **Revinage®** produces significant results in reducing wrinkles in only 28 days (Figure 33).

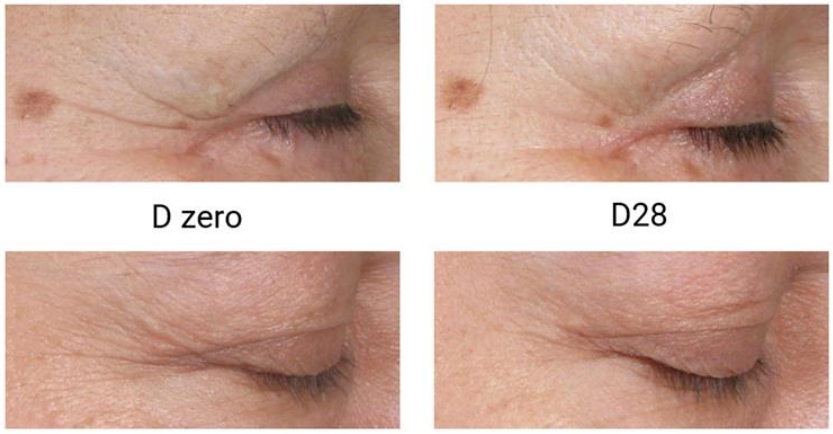


Figure 33. Revinage® effect on the wrinkles of the facial skin (periorbital region) after 28 days of continuous treatment.

4.6. Instrumental evaluation of cutaneous hydration by Raman Spectroscopy (River Diagnostics Model 3510)

In this study, the hydration profile of the mucous membranes of the lips were evaluated after a single application of the test product through Raman spectroscopy after 2 and 4 hours. The proposed parameters were used to learn the effectiveness of the active and thus, its contribution to cutaneous metabolism, related to semi-mucous/skin hydration (lips) in particular.

Three factors were evaluated: the formation of NMF (natural moisturizing factor), ceramides-3 and cholesterol, three important compounds related to the barrier function and moisturizing of the skin. The NMF is a highly effective humectant that is synthesized and located within the stratum corneum. It mainly consists of amino acids and derivatives, PCA (carboxylic pyrrolidone acid), minerals, urea, sugars and peptides[31]. This highly hygroscopic mixture helps to maintain hydration and consequently the function of the skin barrier[31].

The lipid composition of the cutaneous barrier can vary quantitatively between individuals and parts of the body, but is mainly comprised of ceramides (50%), cholesterol (25%) and free fatty acids (10%)[32]. The lipids present in the skin barrier are essential to its operation as they reduce transepidermal water loss and altered scaling[32].

The lipstick containing 1% **Revinage®** significantly stimulated the production of NMF, ceramide-3 and cholesterol, featuring excellent efficiency in key factors for maintaining the integrity of the cutaneous barrier as well as its hydration (Table 2).

Table 2. Stimulation of hydration factors and skin barrier by **Revinage®**

Compound	2 h	4 h
NMF	92.78%	-
Ceramide-3	33%	55.86%
Cholesterol	-	12.25%

4.7. Cutaneous Permeation by Raman Spectroscopy (River Diagnostics Model 3510)

The evaluation of cutaneous permeation of **Revinage®** was carried out in the formula below:

UNIOX A		4.00%
CHEMYLAN L		2.00%
Cetostearyl alcohol		2.00%
Isonanoato of Isonanila		2.00%
CHEMYNOL		0.50%
Disodium EDTA		0.20%
REVINAGE®		**3.00%**
Vitamin E		0.20%
Glycerin		2.00%
Deionized Water	qsp	100.00%

Revinage® demonstrated that the peak of permeation of the active (based on the evaluation of the phytol) was gradually reached 4 h after the application of the formulation, where 65% of Phytol was found below the corneal

stratum (1-60 µm) Table 3). This result demonstrates that **Revinage®** reaches the deepest layers of the skin, acting effectively for the treatment of cutaneous aging.

Tabela 3. Revinage® permeation

Samples	% of active permeation between 1-60µm (relative to phytol concentration)					
	30min	**1h**	**2h**	**4h**	**18h**	**24h**
Placebo	0%	0%	0%	0%	0%	0%
Revinage® Cream	8.12%	29.3%	62.17%	65.15%	9.13%	2.21%

5. Other Tests

Despite presenting fewer side effects than retinoic acid, retinol is extremely unstable mainly when exposed to light and oxygen[28-33]. A comparison study between retinol and **Revinage®** was conducted to compare its stability when exposed to photodegradation (6 hours under UV radiation in a solar simulation chamber) and oxidation (3% peroxide for 15 hours at 30°C). Subsequently, the ingredients were analyzed by high-efficiency liquid chromatography (HPLC) and compared to the material not subjected to stress.

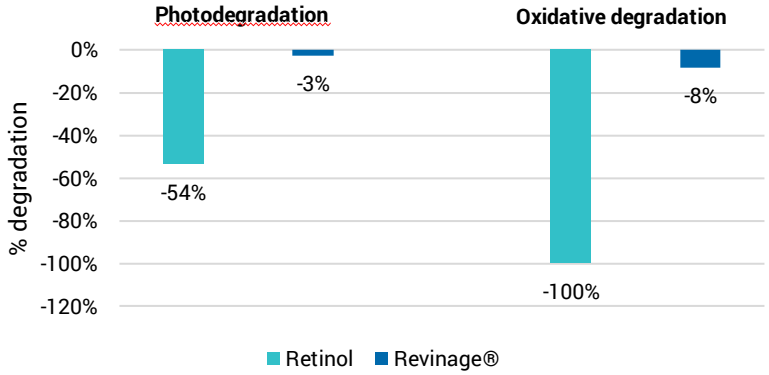


Figure 34. Comparative stability between **Revinage®** and Retinol subjected to photodegradation by 6 h and peroxide at 3% per 15 h at 30ºC.

Revinage® has proved to be far superior to retinol in the two conditions, and much more stable when exposed to light and oxidizing agents. Still, according to the long-term stability studies (shelf-life – 24 months), **Revinage®** is a stable ingredient under normal storage conditions, when there was no variation of the phytol concentration in its composition throughout the study period.

Application

Cream, lotion, non-transparent gel, gel-cream, serum and oil for the treatment of aging and photoaging. Not applicable to hydro alcoholic formulation.

Revinage® must be incorporated into the oily phase up to 85ºC.

Suggested pH: 5.5 to 7.0.

Stability and Compatibility

Solubility: Soluble in oil. It should be incorporated into the oily phase of the emulsion.

Incompatibility: So far, incompatibilities for **Revinage®** have not been evidenced.

Concentration of Use

From 1.0 to 2.0% (w/w)

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