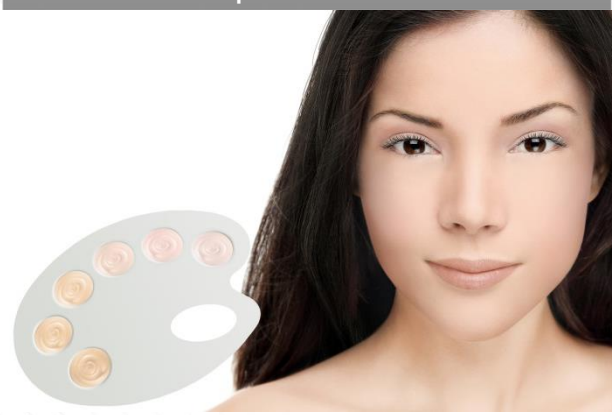
Technical Report



Product

BRIGHLETTE™ *marine ingredient*

Date

April 2014

Revision

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Contents

Need for an even skin tone

The main determinant of the **coloration of the skin** is the pigmentation provided by **melanin**. This substance has an important role in protecting the skin from damage induced by ultraviolet (UV) radiation, but it also determines the appearance of the skin, influencing its tone and luminosity.

The process of aging and the UV damage often related with it are associated with changes in pigmentation. Melanin can accumulate irregularly causing the appearance of areas of focal **hyperpigmentation**. With time these areas increase in number and size and their contrast with normally pigmented skin appears more evident, reducing uniformity of the skin tone. This poses an important esthetic problem, as skin homogeneity is known to influence perception of **attractiveness and youthfulness** [1, 2].

One of the most noticeable alterations are **solar lentigines**, also known as sun or age spots, typically found in UV-exposed areas for instance the face or the dorsum of the hand [3]. Additionally other factors, such as hormonal changes or inflammation can lead to altered pigmentation [3].

The **aesthetic ideals** in most cultures involve presenting an **even skin tone**, while in Asia these also implicate a **lighter** complexion. Indeed, the use of skin lighteners has been reported since ancient times in many civilizations, and currently the use of brightening cosmetic ingredients is widespread.

In many societies, there is the cultural perception that a **lighter complexion is more desirable** and a paler skin has been traditionally associated with high society [4]. On the other hand, the first **signs of aging** appearing in Asian skin, even before wrinkles, are dark spots or hyperpigmented areas, coming out despite little sun exposure [5]. Therefore, the common desire is to brighten and even the skin tone to decrease signs of aging and show a lighter and more attractive look [4].

In **western countries** the aspirations regarding skin tone are to reduce the unwanted pigmented areas and prevent the formation of new ones to eventually **enhance evenness**. In addition, improving general radiance and luminosity are interesting to achieve a luminous and more attractive appearance.

Either to increase lightness or to improve evenness, there is a global demand to correct the skin tone.

Production of melanin in the skin

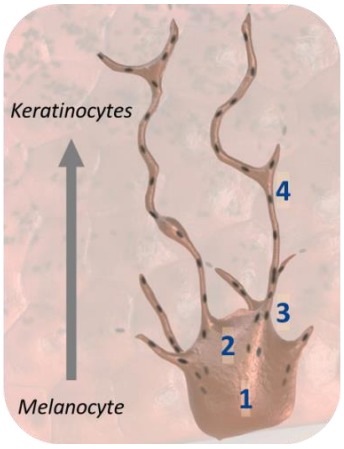
The process by which melanin is synthesized and distributed in the epidermis to provide pigmentation is complex and involves multiple steps.

The production of melanin, also known as **melanogenesis**, takes place in highly specialized cells, called **melanocytes** [3]. These cells are located in the basal layer of the epidermis and spread many dendritic extensions (or dendrites) that contact with keratinocytes of the lower epidermis [6]. So, each melanocyte is in contact with 30-40 keratinocytes, composing the **epidermal melanin unit** [3, 6].

There is an extensive crosstalk between keratinocytes and the melanocyte in the epidermal melanin unit, with intricate cellular and molecular interactions between them that determine pigmentation [7]. So, for instance, UV radiation stimulates keratinocytes to release diverse signaling factors that act on melanocytes to induce melanogenesis [7].

Melanocytes synthesize melanin within membrane-bound organelles, known as **melanosomes**. Once these vesicles are filled with melanin pigment, they are transported along melanocyte dendrites and **transferred to keratinocytes**. In keratinocytes, melanin absorbs and scatters UV light, providing certain photoprotection to the skin [3, 8].

The **degree of pigmentation** and therefore the visible color of the skin are determined by several factors, basically occurring throughout the whole process of melanin production and spreading. The most important factor is the **activity of melanocytes**, which is directly related to the quantity of melanin produced and the number of melanosomes. The eventual level of pigmentation is also affected by the efficiency of melanosome transfer and the distribution of melanosomes within keratinocytes [3, 6, 9].



1. Activation of melanocytes
2. Melanosome biogenesis and maturation
3. Synthesis of melanin in melanosomes
4. Transport and transfer of melanosomes

Fig. 1. Epidermal melanin unit and the production of melanin in the skin.

The production and distribution of melanin in the skin is modulated at different stages.

Skin pigmentation involves multiple steps

The process of leading to melanin deposition in the skin includes several phases. First, the **activation of the melanocyte** that results in the transcription of genes required for melanogenesis. Then, **melanosomes** are formed and **melanin** is synthesized within them. Finally, melanosomes are transported to the tips of melanocyte dendrites and **transferred** to keratinocytes [10].

Activation of melanocytes

Melanocyte functions and the process of melanogenesis are controlled by **several signaling pathways**. These intracellular routes are activated by the binding of ligands to specific receptors in the melanocyte membrane and, after multiple steps, all of them converge on the activation of the microphthalmia-associated transcription factor (MITF), a critical regulator of melanogenesis but also dendricity, proliferation and survival of melanocytes [10, 11, 12].

Endothelins are essential proteins for melanocyte development. The most relevant is endothelin-1, which is secreted by keratinocytes after exposure to UV radiation. It binds to the endothelin receptor B (ENDRB) on melanocytes membrane leading, to the transcription of MITF [3, 12]. This transcription factor is also upregulated by the **Wnt family of proteins**. These are secreted glycoproteins that regulate numerous cell functions. In melanocytes, Wnt-1 binds to and activates Frizzled (FZD) receptors and, through subsequent intracellular signaling, results in MITF activation [10]. Wnt is overexpressed in solar lentigines and is thought to contribute to their development [13]. Dickkopf-related protein 1 (DKK1) is an inhibitor of the Wnt pathway that is for the regulation of melanocyte function [11].

Once activated, MITF controls melanogenesis by stimulating the **transcription of several melanogenic elements**, which include the enzymes responsible for melanin synthesis but also proteins that are required for the formation and maturation of melanosomes [3, 10, 12].

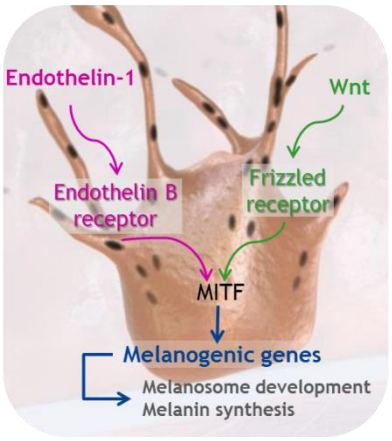


Fig. 2. Some of the signalling pathways that mediate melanocyte activation.

Melanosome biogenesis and maturation

Genes induced by MITF are translated into proteins that lead to the formation of **melanosomes**, the separate cytoplasmic organelles where the biochemical pathway responsible for melanin synthesis takes place [3].

Melanosomes typically follow **four maturation stages** (I-IV), according to their structure and the quantity and arrangement of melanin produced [3]. In early melanosomes (stages I and II) an internal fibrillar matrix is formed and melanin-synthesizing enzymes are incorporated [8, 11, 14]. In late melanosomes (stages III and IV) melanin is synthesized and deposited on

the matrix fibrils, resulting in vesicles completely filled with pigment [11, 14]. Then, the activity of melanin-synthesizing enzymes is lost and melanosomes can be transported [3].

Apart from the enzymes, melanosomes contain **structural fibrillar proteins** that are essential for their formation and maturation [6, 11]. Pmel17 is an important structural component of melanosomes, playing a role in the production of internal fibrils [11]. **Melan-A** (also known as MART-1) is a melanosome-specific protein localized in melanosomes membrane that is highly enriched in early melanosomes, suggesting that it plays a role in their biogenesis [15, 16]. Melan-A forms a complex with Pmel17 to control the function of the latter, and thus modulates melanosome formation and maturation [11, 15].

Synthesis of melanin in melanosomes

Human melanocytes produce two chemically distinct kinds of melanins: **black-brown eumelanin** and **yellow-reddish pheomelanin** [3, 6]. The skin contains a mixture of both and their ratio determines visible pigmentation [8]. Eumelanin is the major type in individuals with dark skin and hair, while pheomelanin is abundant in individuals with red hair and fair skin [8].

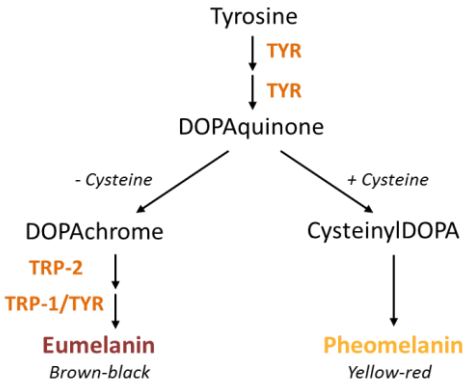


Fig. 3. Melanin biosynthesis.

Three enzymes are absolutely required for melanin synthesis within melanosomes [3]. The type of melanin produced is determined by the availability of substrates (e.g. cysteine) and the activity of the melanogenic enzymes [6, 8]. **Tyrosinase** (TYR) is the rate-limiting enzyme and catalyzes the first two steps of the process, required for the synthesis of both types of melanin [9]. **Tyrosinase-related protein-1 and -2** (TRP-1 and TRP-2) collaborate with TYR to synthesize eumelanin. TRP-2 can process dihydroxyphenylalanine (DOPA)chrome in the absence of cysteine [9]. In the subsequent step, TRP-1 and tyrosinase catalyze eumelanin production [6, 10].

Transport and transfer of melanosomes

When melanin synthesis is completed, **melanosomes are transported along the dendrites** in a movement controlled by elements of the cytoskeleton [8, 9, 11, 13]. Once they reach dendrite tips, melanosomes are transferred to keratinocytes. Various transfer mechanisms are thought to occur, including **phagocytosis of melanosomes by keratinocytes** [8, 12]. **DKK1** is involved in the transfer of melanosomes, as it inhibits phagocytosis of the melanin-containing vesicles by keratinocytes [17].

Activation of melanocytes leads to melanin synthesis in melanosomes that are then transferred to keratinocytes.

Repair DNA damage in the skin

UV radiation is a key factor in the regulation of melanogenesis, as it boosts the levels of melanin in the skin to enhance protection. A complementary mechanism of defense is the repair of damage when it occurs. Being so exposed to UV light, **repair of DNA damage in the skin** is an essential to maintain a youthful appearance.

All cells in the skin, including keratinocytes and melanocytes, need to efficiently remove DNA lesions to preserve all their functions correctly. There are various **DNA repair pathways** in every cell, each consisting of a cascade with numerous proteins that work to repair specific types of lesions (e.g. single or double chain breaks, crosslinks, adducts) [18].

Nucleotide excision repair (NER) is one of the most versatile and flexible repair systems and can counteract the deleterious effects of a multitude of DNA lesions [19]. Importantly, the NER system is critical for **correcting UV-induced DNA damage** [20]. This repair pathway comprises around 30 factors that work to remove the damaged part of the DNA and restore the original sequence using the opposite strand of the DNA as a template [20, 21].

Basically, the NER system consists of four main steps. The first step is the **recognition of DNA damage**. RNA polymerase II, which consists of 12 subunits (A-L), normally transcribes genes from the DNA sequence to induce their expression. When it finds a lesion, it gets stalled becoming a recognition signal for the excision-repair cross-complementing proteins 6 and 8 (ERCC6 and ERCC8) that act to recruit repair proteins needed for the subsequent steps [20, 21].

Then, another set of proteins, including helicases, **unwind the DNA double helix** around the site of the lesion to allow the access of repair proteins.

In the next step, the damaged part of the DNA is removed. The endonuclease complexes xeroderma pigmentosum, complementation group G (XPG) and xeroderma pigmentosum, complementation group F (XPF)-ERCC1 bind to the DNA and make two incisions **releasing the damaged DNA** [20, 21].

Lastly, the gap is filled by various DNA polymerases, the proliferating cell nuclear antigen (PCNA) and the replication factor C (RFC). All these proteins **synthesize new DNA** complementary to the undamaged strand [21]. Finally DNA is sealed and repaired [20, 21].

DNA repair is essential to prevent damage accumulation and preserve skin properties.

brighlette™ marine ingredient

Biointec™ blue biotechnology

BRIGHLETTE™ *marine ingredient*, step by step to perfectly even skin tone

BRIGHLETTE™ *marine ingredient* is an extract obtained by biotechnology from a marine microorganism isolated from the southwest coast of Tenerife (Canary Islands archipelago, Spain). It acts on **multiple stages of the pigmentation process** to consistently reduce the deposition of melanin in the epidermis.

In vitro, the active ingredient regulates **gene expression of melanogenic proteins** that normally control melanin production and distribution in the epidermis. Apart from reducing gene expression of melanin-synthesizing enzymes, it also reduces the **expression of tyrosinase protein and its activity**, minimizing the potential to produce pigment.

BRIGHLETTE™ *marine ingredient* also targets the melanosome-specific protein melan-A to **interfere with the maturation of the organelles where melanin is produced**. It was also confirmed that the extract **reduces the production of melanin** in melanocyte cultures. Additionally, the active ingredient shows an **inhibitory effect on phagocytosis**, the mechanism by which keratinocytes incorporate melanosomes.

In a skin model of induced hyper-pigmentation related to age spots, BRIGHLETTE™ *marine ingredient* is able to **prevent excessive melanin** accumulation. In addition, the expression of **DNA repair pathways is induced**, to counteract a potential decrease in protection.

As assessed through an *in vivo* test, the extract provides a **brightening effect** (increasing L* and ITA^0) and **homogenizes skin complexion** by reducing contrast of dark spots and their size and melanin content.

BRIGHLETTE™ *marine ingredient* brightens the skin, especially in dark spots, greatly increasing evenness.

In vitro efficacy

MODULATION OF THE EXPRESSION OF MELANOGENIC GENES

Microarray analysis of gene expression was performed to verify its modulation by the active ingredient on melanocytes.

Human epidermal melanocytes from neonatal, darkly-pigmented skin were treated with 10 µg/mL BRIGHLETTE™ *marine ingredient concentrate* for 13 days, repeating treatment on days 3, 6, 8 and 10. Non-treated cells are used as controls. Then, the cells were lysed and RNA was extracted and purified.

A microarray was performed using an ASurePrint G3 Human Gene Expression Microarray v2 platform. After normalization and analysis of the microarray data, fold induction in the expression of selected genes with respect to the control values was calculated.

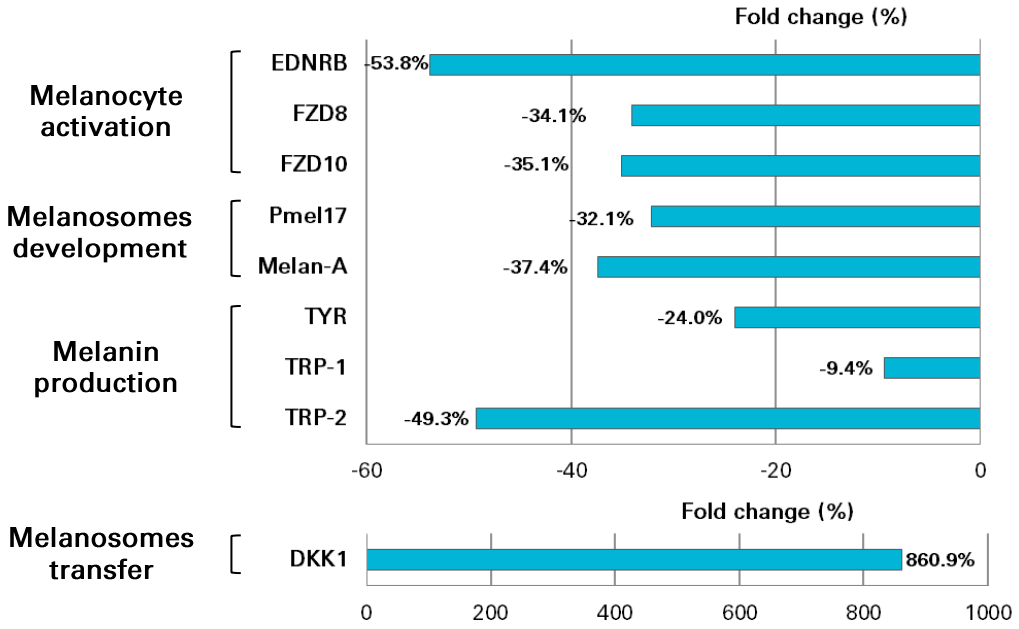


Fig. 4. Regulated genes involved in the pigmentation pathway.

The marine ingredient **modulates genes** corresponding to melanogenic **signaling pathways**, **melanosomal proteins** and **melanin-synthesizing enzymes**.

Several genes of the pigmentation process are regulated by BRIGHLETTE™ *marine ingredient*.

DECREASE IN MELANOSOMES MATURATION

Co-cultures of keratinocytes and melanocytes, the two cell types of the melanin epidermal unit, were treated with the active ingredient to assess changes induced in the melanosome-specific marker melan-A, which is needed for the formation and maturation of melanosomes.

Human epidermal melanocytes from neonatal, darkly-pigmented skin were seeded with primary human keratinocytes to establish a co-culture. Then, the cells were incubated for 48 h with different treatments: 4 µg/mL forskolin (to induce melanosome maturation), 71 µg/mL kojic acid (positive control), 10 µg/mL BRIGHLETTE™ *marine ingredient concentrate* or non-treated (control).

Then, melan-A protein was stained by immunofluorescence. An antibody with a green fluorescence emission dye was used to detect melan-A and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

Images were captured using a fluorescence microscope. For each fluorescence image, integrated density values were normalized by the number of nuclei. These data, indicating melan-A protein levels, were then normalized by the mean values of control cells.

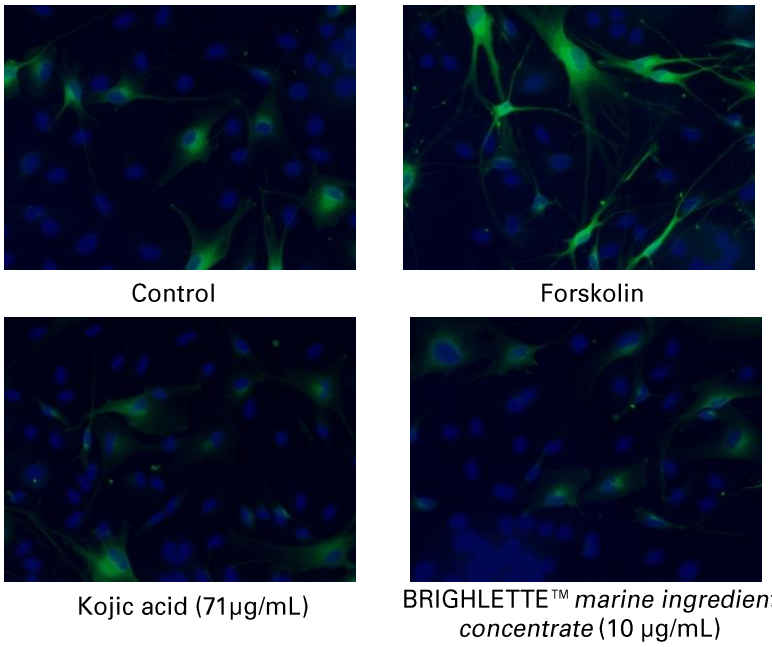


Fig.7. Immunofluorescence images of melan-A protein (green). Keratinocytes and melanocytes nuclei are seen as blue.

A **decrease in the levels of melan-A** protein was observed in melanocyte-keratinocyte co-cultures after treatment with BRIGHLETTE™ *marine ingredient*.

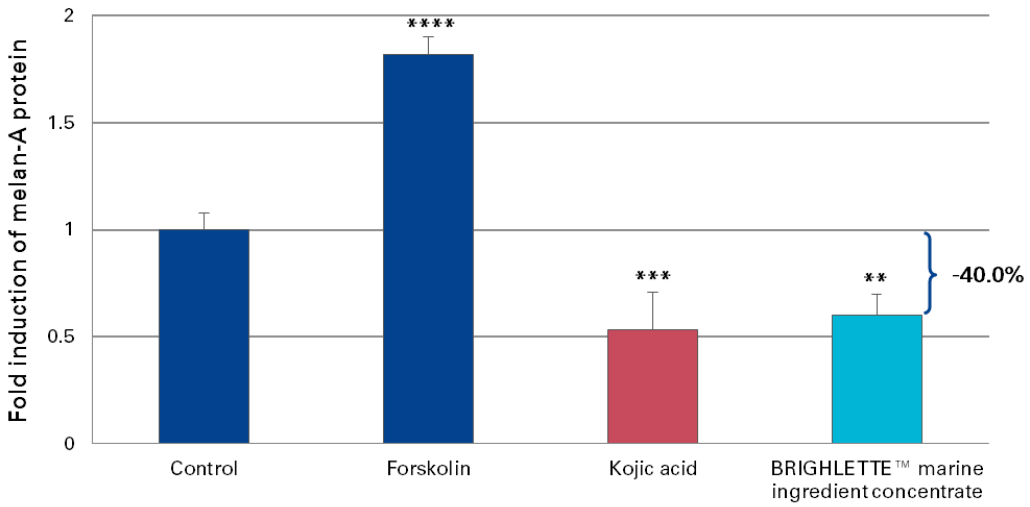


Fig. 8. Fold-induction of melan-A protein (**p<0.01, ***p<0.001, ****p<0.0001).

The treatment with the active ingredient **reduced protein** levels of **melan-A by 40.0%**.

BRIGHLETTE™ *marine ingredient* interferes with the development of melanosomes.

REDUCTION IN TYROSINASE PROTEIN EXPRESSION

The ability of BRIGHLETTE™ *marine ingredient* to modulate tyrosinase protein levels found within melanocytes was studied as this enzyme is absolutely required for melanin synthesis in melanosomes.

Human epidermal melanocytes from neonatal, darkly-pigmented skin were cultured for 13 days with the active ingredient concentrate or only with medium (control). The treatments were repeated on days 3, 6, 8 and 10.

After the treatments, the melanocytes were fixed and a cell-based enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the quantity of tyrosinase protein present in the cells. After incubations with the corresponding antibodies, the colorimetric reaction was detected by measuring absorbance at 450 nm.

Tyrosinase absorbance values were normalized by those of an internal control, allowing the calculation of tyrosinase relative amounts.

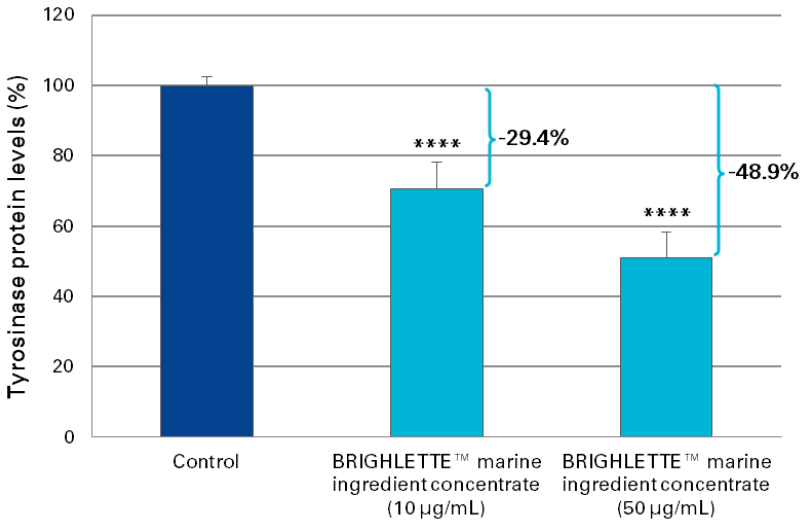


Fig. 5. Tyrosinase protein levels in melanocytes (****p<0.0001).

The active ingredient **reduced** the amount of **tyrosinase protein within melanocytes** by up to 48.9%

BRIGHLETTE™ *marine ingredient* effectively decreases protein tyrosinase.

INHIBITION OF HUMAN TYROSINASE ACTIVITY

The inhibitory effect of BRIGHLETTE™ *marine ingredient* on the activity of human tyrosinase was evaluated using an enzyme assay.

To assess changes in the activity of this enzyme, a tyrosinase assay kit that measures the formation of a DOPAchrome complex that absorbs at 490 nm was used.

First, a reaction mix was incorporated into microplate wells and, then kojic acid (positive control) or BRIGHLETTE™ *marine ingredient concentrate* were added at different concentrations. After loading the tyrosinase enzyme, absorbance was read at 490 nm, every minute for 20 minutes and at 37 °C using a microtiter plate reader.

Data was normalized with respect to the control absorbance values, obtained without the active ingredient or kojic acid.

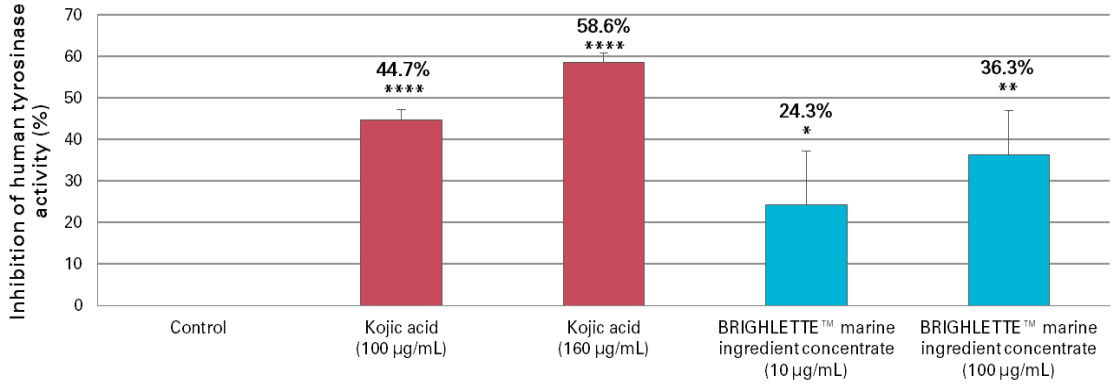


Fig. 6. Inhibition of human tyrosinase activity with respect to control (*p<0.05, **p<0.01, ****p<0.0001).

The active ingredient **diminished human tyrosinase** activity in a statistically significant manner and up to 36.3% indicating its potential to decrease melanin synthesis.

BRIGHLETTE™ *marine ingredient* has an inhibitory activity on human tyrosinase.

MELANIN PRODUCTION DECLINE IN MELANOCYTES

This test was performed to assess the effects of the active ingredient on the quantity of melanin pigment produced by melanocytes

Human epidermal melanocytes from neonatal, darkly-pigmented skin were treated with different concentrations of BRIGHLETTE™ *marine ingredient concentrate*, while non-treated cells were used as a control. The cultures were kept for 13 days, repeating the treatments every 2-3 days.

Then, the cells were counted, and melanin was extracted from them and its absorbance at 450 nm determined. Melanin levels in each sample were quantified from a standard curve plotted from known concentrations of synthetic melanin and normalized by cell number in each condition.

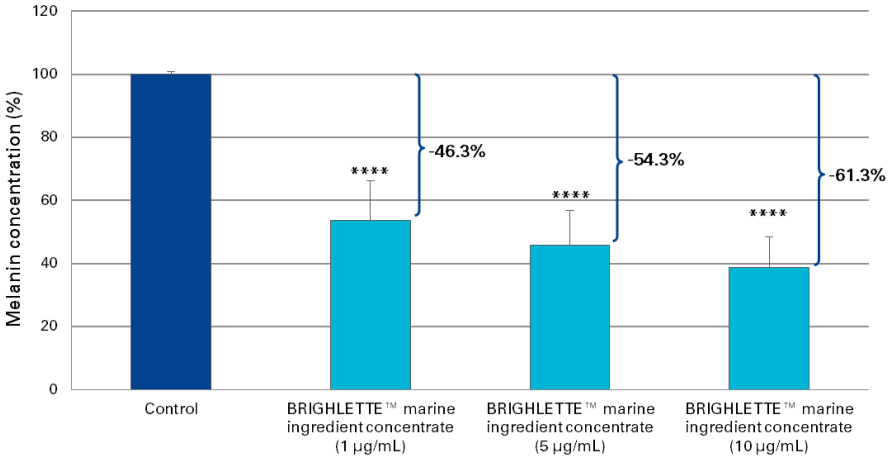


Fig. 9. Percentage of melanin after the treatment (****p<0.0001).

Control

BRIGHLETTE™ *marine ingredient concentrate* (10 µg/mL)

Fig. 10. Human melanocytes at the end of the treatment.

The amount of **melanin produced** by melanocytes **dropped by 61.3%**, a decrease that was also visible in the cell cultures.

BRIGHLETTE™ *marine ingredient* diminishes melanin production in melanocytes.

MODULATION OF PHAGOCYTOSIS IN KERATINOCYTES

The supernatants of melanocytes incubated with the active ingredient were used to treat keratinocytes and evaluate the effect of melanocyte-secreted factors on the modulation of phagocytosis in keratinocytes, the process by which these cells can take up melanosomes.

Human epidermal keratinocytes (HEKa) were incubated for 30 min with medium alone (non-treated control) or with 100 ng/mL synthetic DKK1 as a positive control for phagocytosis inhibition.

Separately, human epidermal melanocytes from neonatal, darkly-pigmented skin were incubated with medium alone (control) or 1 µg/mL BRIGHLETTE™ *marine ingredient concentrate* for 13 days, repeating treatments on days 3, 6, 8 and 10. Melanocyte supernatants were then collected and used to treat HEKa for 30 min. HEKa incubated with supernatants from control melanocytes were used as a supernatant-treated control.

After the treatments, the cells were incubated for 4 h with fluorescent microspheres (0.5 µm diameter) to allow their phagocytosis by HEKa.

Finally, the cultures were washed to remove non-internalized spheres and internalization of microspheres by the cells was observed. The cells were stained with fluorescently labelled phalloidin to reveal their outline and images were captured using confocal fluorescence microscopy. To quantify microsphere uptake, the number of spheres was normalized by the cell number in each fluorescence image.

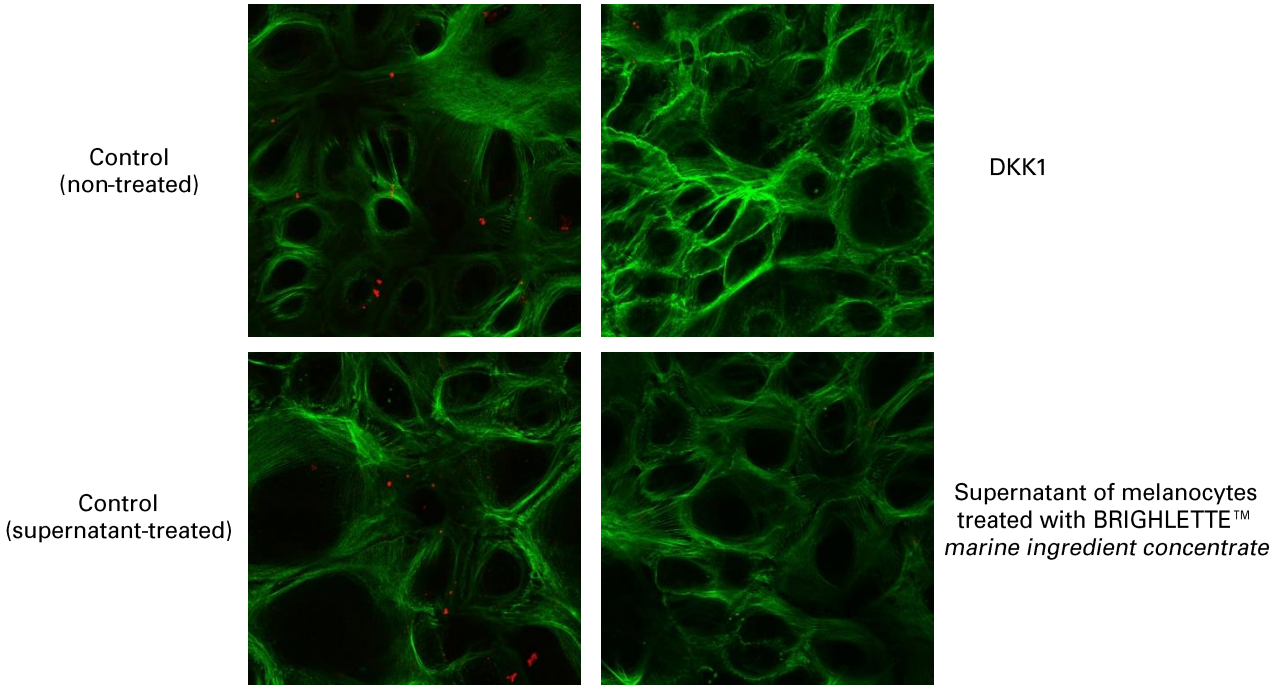


Fig. 11. Images of keratinocytes after the different treatments. Red indicates the microspheres and green the cell outlines.

A **decrease in internalization** of spheres was observed in human **keratinocytes** after incubation with DKK1 or with supernatants from **melanocytes treated with BRIGHLETTE™ *marine ingredient***.

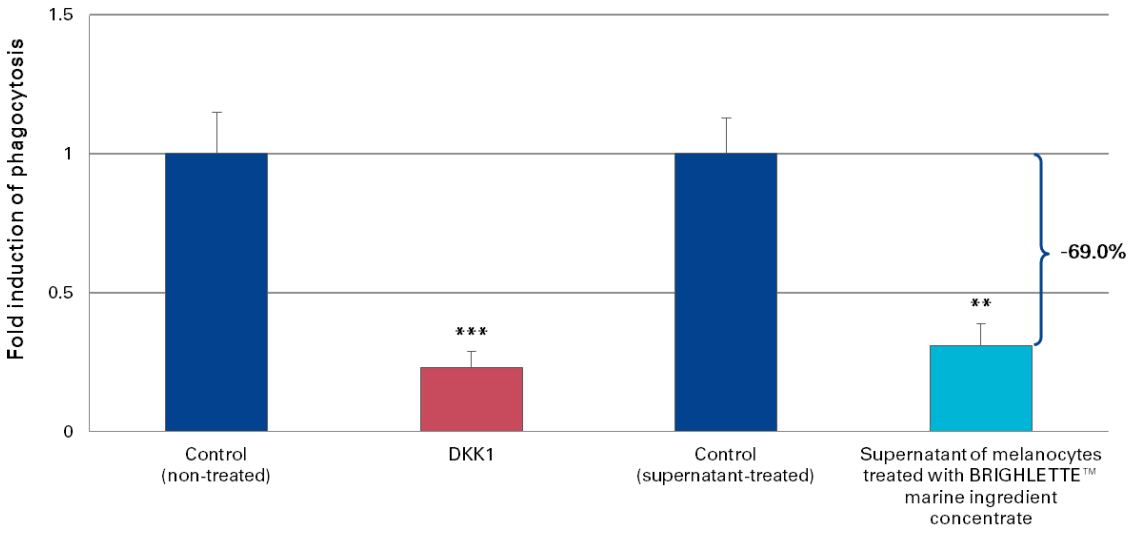


Fig. 12. Fold-induction of phagocytosis in keratinocytes (**p<0.01, ***p<0.001).

Treatment of melanocytes with BRIGHLETTE™ *marine ingredient* **reduced by 69.0%** the mechanism by with keratinocytes can incorporate melanin-containing melanosomes. This effect may be mediated through the induction of DKK1 in melanocytes by the extract.

BRIGHLETTE™ *marine ingredient* shows an inhibitory effect on phagocytosis in keratinocytes.

MELANOGENESIS INHIBITION ON RECONSTRUCTED EPIDERMIS

The ability of the active ingredient to prevent melanogenesis and hyperpigmentation was assessed in an epidermis model treated with Wnt-1, an activator of melanocytes that is involved in the development of age spots.

Reconstructed human pigmented epidermis (phototype IV) was incubated with different products, repeating the treatments every day for 5 days. The treatments included Wnt-1 (200 ng/mL) to induce hyper-pigmentation and Wnt-1 with 100 or 200 µg/mL BRIGHLETTE™ *marine ingredient concentrate*. Epidermis models incubated with medium alone were used as controls.

After the treatments, tissue sections were made and a Fontana-Masson stain was performed to detect melanin within the epidermis. Images of the stained tissue sections were captured using an optical microscope. Stained areas, corresponding to melanin, were quantified in each image and normalized by the mean values of control.

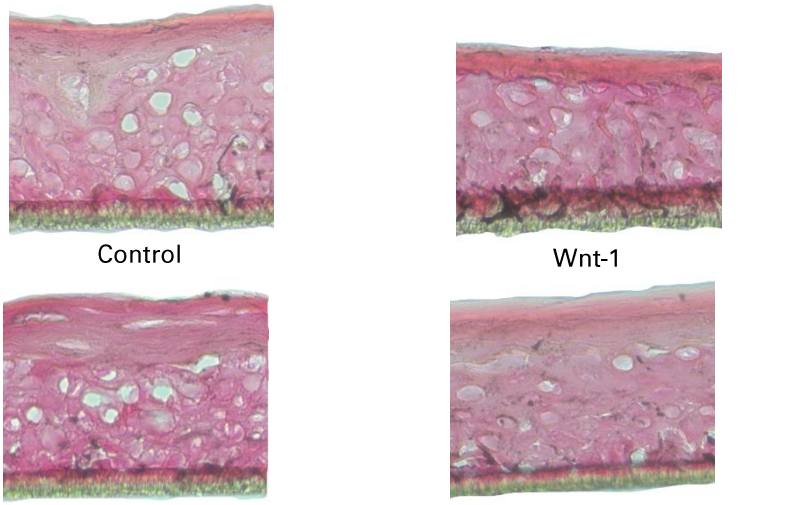


Fig. 13. Images of reconstructed epidermis after 5 days of treatment, showing melanin staining in black.

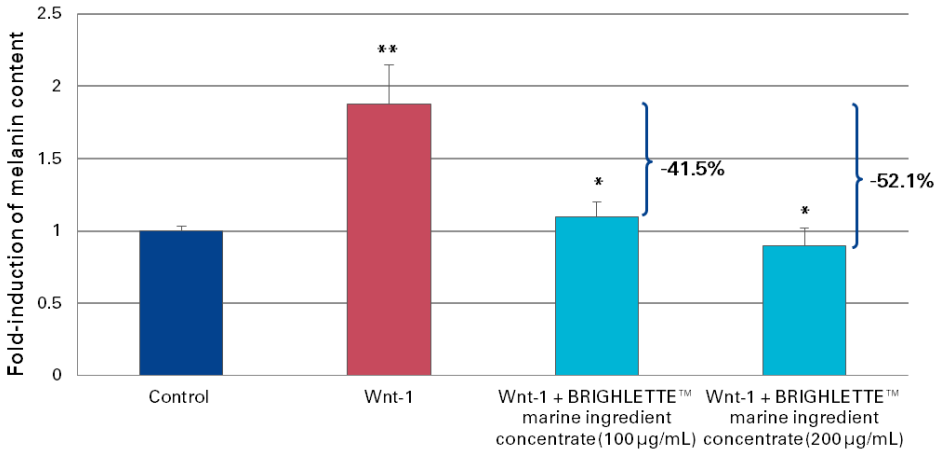


Fig. 14. Fold induction of melanin content in reconstructed epidermis (*p<0.05, **p<0.01).

BRIGHLETTE™ *marine ingredient* prevents hyperpigmentation related to age spots.

DNA REPAIR PATHWAY ACTIVATION

Microarray analysis was used to detect the genes that were regulated by BRIGHLETTE™ *marine ingredient* in a skin model.

Reconstituted epidermis (phototype IV) was treated with medium alone (control) or 10 µg/mL BRIGHLETTE™ *marine ingredient concentrate* for 48 h, repeating treatment every 24h. Then, RNA was extracted and purified from the tissue and its quality verified.

A microarray was performed using an ASurePrint G3 Human Gene Expression Microarray v2 platform. After normalization and analysis of the microarray data, fold induction in the expression of selected genes with respect to the control values was calculated.

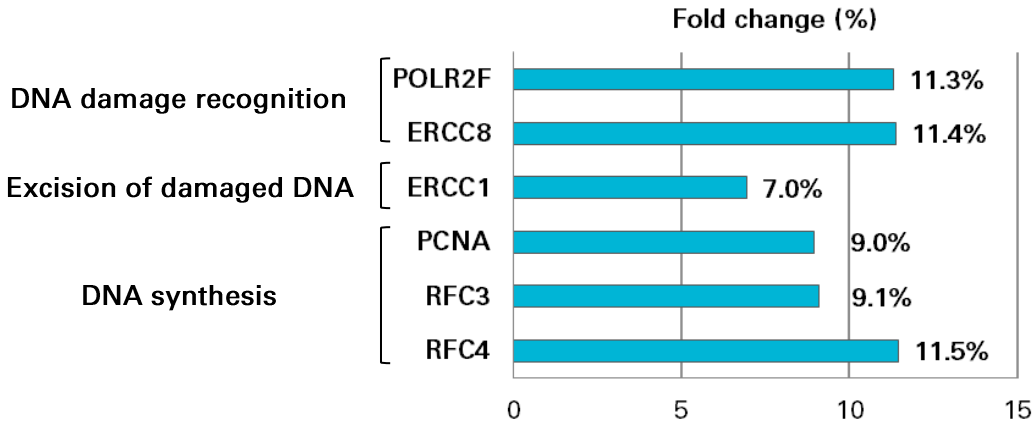


Fig. 15. Percentage of fold induction of overexpressed genes of the NER DNA repair pathway.

The active ingredient **upregulated** components of the **NER DNA repair pathway in the epidermis**, necessary due to the loss of protective pigment.

BRIGHLETTE™ *marine ingredient* enhances gene expression to repair DNA in the skin.

In vivo efficacy

BRIGHTENING AND EVENING THE SKIN COMPLEXION

A group of 22 Asian female volunteers, between 38-53 years old and with hyperpigmented regions on the skin applied a cream containing 2% BRIGHLETTE™ *marine ingredient* or a placebo cream twice a day for 8 weeks.

INCREASE OF L* AND ITA°

The skin color was assessed on hyperpigmented and non-hyperpigmented regions of the subject's cheek before and after 8 weeks of treatment.

A spectrophotometer was used to assess, the luminance parameter (L*), which represents relative brightness, from total darkness (L*=0) to absolute white (L*=100). The individual typological angle (ITA°) was calculated from L* to have a quantification of the changes in the natural skin colors.

L* and ITA° parameters were considered for the assessment of the brightening effect: the lighter the skin, the higher L* and ITA° values. The skin contrast between hyper-pigmented and non-hyperpigmented regions was calculated for L* and ITA°, defined as the difference between the value in the non-hyperpigmented region and in the hyper-pigmented region.

Additionally, digital photographs were taken using a facial imaging system before and after the treatment. Images were captured with different lights.

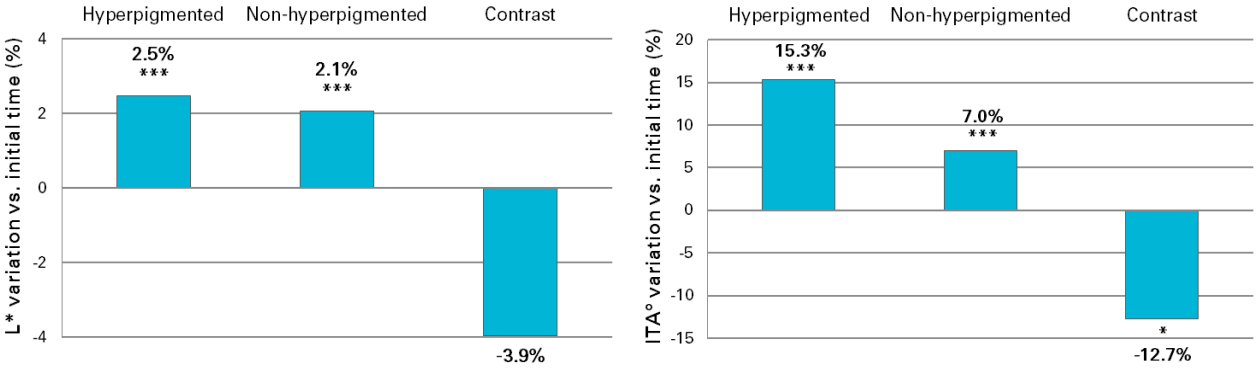


Fig. 16. Evolution of L* and ITA° parameters versus the initial time (*p<0.05, ***p<0.001).

L* and ITA° increased in normally-pigmented skin, revealing a **skin brightening effect**. In hyperpigmented skin, this effect was even greater and the ITA° **contrast between dark spots and normally pigmented skin decreased by 12.7%**, meaning a homogenization in the skin tone.

BRIGHLETTE™ *marine ingredient* increased ITA° by 15.3% and L* by 2.5% in hyperpigmented areas.

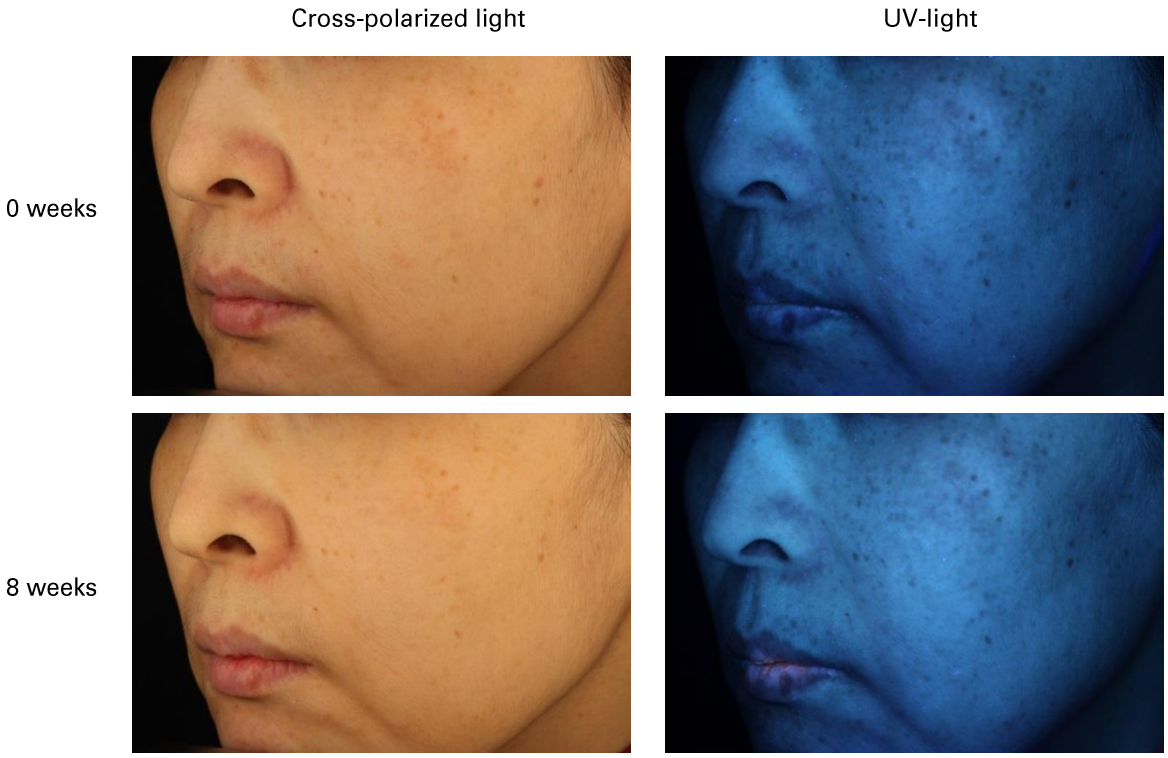


Fig. 17. Overall skin pigmentation of a volunteer before and after the treatment.

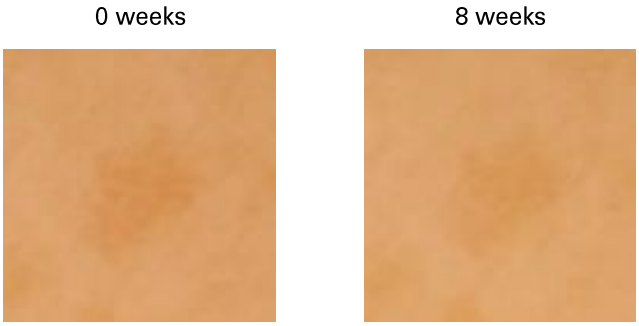


Fig. 18. Hyperpigmented spot in the skin of a volunteer under cross polarized light.

With BRIGHLETTE™ *marine ingredient* there is an overall brightening and homogenization of the skin.

SIZE REDUCTION OF HYPERPIGMENTED SPOTS

The variation in the size of hyperpigmented spots was assessed by analysis of digital photographs taken with a facial imaging system before and after 8 weeks of treatment. Measurement of the area of hyperpigmented regions was performed using appropriate image analysis software.

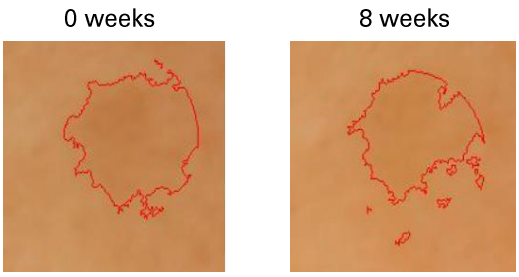


Fig. 19. Analysis image on a hyperpigmented region.

The **area occupied by hyperpigmented regions** revealed a statistically significant **reduction of 6.9%** at the end of the treatment. This reduction in the size of dark spots was 19.5% higher compared to the placebo treatment.

BRIGHLETTE™ *marine ingredient* reduces the size and visibility of dark spots.

EFFECT ON MELANIN CONTENT IN DARK SPOTS

The amount of melanin on hyperpigmented regions was measured by reflectance confocal microscopy.

Confocal microscopy allows obtaining an *in vivo*, non-invasive view into the skin. To obtain a melanin image, a near-infrared laser (830 nm) is directed onto the individual skin sections where it is reflected. Melanin acts as natural contrast agent due to its relatively high refractive index.

The device generates images of skin in an optimal quality that allow quantification of melanin.

Melanin images of 2 volunteers were analyzed as intensity on hyperpigmented region by using adequate image analysis software.

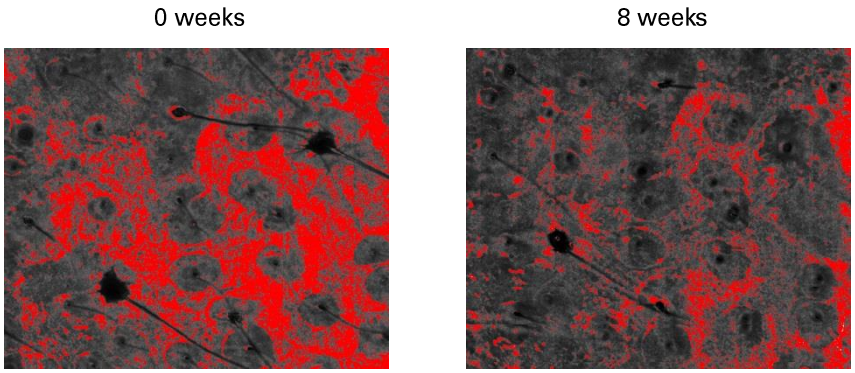


Fig. 20. Visualization of melanin before and after the treatment in confocal microscopy images.

The intensity of **melanin** in hyperpigmented regions **decreased by** an average of **61.1%** at the end of the treatment.

The quantity of melanin in dark spots is notably diminished.

brighlette™
marine ingredient

Biointec™
blue biotechnology

Cosmetic properties

BRIGHLETTE™ *marine ingredient*:

- biotechnological marine ingredient that acts on **multiple stages of the pigmentation process**.
- modulated the expression of **melanogenic genes** and induces **DNA repair** related genes.
- diminished the amount of human **tyrosinase protein** by up to 48.9% and its **activity** by 36.3%.
- reduced **melan-A** protein levels (40.0%), thus interfering with **development** of **melanosomes**.
- decreased the amount of **melanin** pigment produced by melanocytes in culture by 61.3%.
- acting on melanocytes, resulted in a 69.0% lower uptake of particles (**phagocytosis**) in keratinocytes.
- neutralized Wnt-induced **hyperpigmentation** (related to age spots) in reconstituted epidermis.
- applied at 2% *in vivo*, provided **brightness** by increasing L* (2.5%) and ITA^0 (15.3%), and decreased **contrast** of dark spots, leading to a great improvement in **evenness**.
- shrank **darks spots area** (6.9%) and decreased their **melanin content** by 61.1%.

Cosmetic applications

BRIGHLETTE™ ***marine ingredient*** can be used in any cosmetic formulation to provide a brighter complexion but also in anti-aging products to even the skin tone by reducing dark spots.

Technical data

INCI NAME OF THE ACTIVE INGREDIENT

Active ingredient	INCI name
BRIGHLETTE™ *marine ingredient*	Plankton Extract

PRESENTATION AND PRESERVATIVE

Solution containing 0.11% Plankton Extract.

Code	Product presentation	Preservative
BI090	BRIGHLETTE™ *marine ingredient*	Preservative free

Application data

PROCESSING

BRIGHLETTE™ *marine ingredient* can be formulated in the aqueous phase of the formulations (emulsions, creams, gels and lotions), in the final step of the manufacturing process. In case of preparing an emulsion, it should be added once the emulsion is formed. It should be provided that the temperature is below 40 °C.

Recommended pH range between 4.0 and 7.0 for BRIGHLETTE™ *marine ingredient*.

INCOMPATIBILITIES

Oxidants and strong electrophiles.

SOLUBILITY

Soluble in water, alcohols and glycols. Insoluble in oils and silicones.

DOSAGE

A dosage of 2% of BRIGHLETTE™ *marine ingredient* is recommended in final cosmetic formulations.

References

1. Huixia Q, Xiaohui L, Chengda Y, Yanlu Z et al. Instrumental and clinical studies of the facial skin tone and pigmentation of Shanghaiese women. Changes induced by age and a cosmetic whitening product. *Int J Cosmet Sci*. 34(1):49-54, 2012.

2. Matts PJ, Fink B, Grammer K, Burquest M. Color homogeneity and visual perception of age, health, and attractiveness of female facial skin. *J Am Acad Dermatol*. 57(6):977-84, 2007.

3. Costin GE, Hearing VJ. Human skin pigmentation: melanocytes modulate skin color in response to stress. *FASEB J*. 21: 976–994, 2007.

4. Saint-Léger D. The colour of the human skin: fruitful science, unsuitable wordings. *Int J Cosmet Sci*. doi: 10.1111/ics.12194, 2014.

5. Farage MA, Miller KW, Elsner P, Maibach HI. Intrinsic and extrinsic factors in skin ageing: a review. *Int J Cosmet Sci*. 30, 87–95, 2008.

6. Bolognia JL, Orlow SJ. Dermatology. 3rd ed. Chapter 65, Melanocyte Biology. Mosby; 2003.

7. Yaar M, Gilchrest BA. Melanocyte Biology: Before, During, and After the Fitzpatrick Era. *J Invest Dermatol*. 122, 27–29, 2004.

8. Cichorek M, Wachulska M, Stasiewicz A, Tyminska A. Skin melanocytes: biology and development. *Postep Derm Alergol*. 1: 30-41, 2013.

9. Ferreira dos Santos I, Lima DF, Magina S. Mechanisms regulating melanogenesis. *An Bras Dermatol*. 88(1):76-83, 2013.

10. Yaar M. Cutaneous Pigmentation in Health and Disease: Novel and Well-Established Players. *J Invest Dermatol*. 133, 11–13, 2013.

11. Yamaguchi Y, Hearing VJ. Physiological factors that regulate skin pigmentation. *Biofactors*. 35(2): 193–199, 2009.

12. Kondo T, Hearing VJ. Update on the regulation of mammalian melanocyte function and skin pigmentation. *Expert Rev Dermatol*. 6(1): 97–108, 2011.

13. Yamada T, Hasegawa S, Inoue Y, Date Y, et al. Accelerated differentiation of melanocyte stem cells contributes to the formation of hyperpigmented maculae. *Experimental Dermatology*. 23, 652–658, 2014.

14. Slominski A, Tobin DJ, Shibahara S, Wortsman. Melanin pigmentation in mammalian skin and its hormonal regulation. J. *Physiol Rev*. 84(4):1155-228, 2004.

15. Hoashi T, Watabe H, Muller J, Yamaguchi Y, et al. MART-1 is required for the function of the melanosomal matrix protein PMEL17/GP100 and the maturation of melanosomes. *J*

Biol Chem. 280(14):14006–16, 2005.

16. Giordano F, Bonetti C, Surace EM, Marigo V, et al. The ocular albinism type 1 (OA1) G-protein-coupled receptor functions with MART-1 at early stages of melanogenesis to control melanosome identity and composition. *Hum Mol Genet*. 18(23):4530-45. 2009.

17. Yamaguchi Y, Morita A, Maeda A, Hearing VJ. Regulation of skin pigmentation and thickness by Dickkopf 1 (DKK1). *J Investig Dermatol Symp Proc*. 14(1):73-5, 2009.

18. Houtgraaf JH, Versmissen J, van der Giessen WJ. A concise review of DNA damage checkpoints and repair in mammalian cells. *Cardiovasc Revasc Med*. 7(3):165-72, 2006.

19. de Laat WL, Jaspers NGJ, Hoeijmakers JHJ. Molecular mechanism of nucleotide excision repair. *Genes & Development*. 13:768–785, 1999.

20. Moriwaki S, Takahashi Y. Photoaging and DNA repair. *J Dermatol Sci*. 50, 169-176, 2008.

21. Budden T, Bowden NA. The Role of Altered Nucleotide Excision Repair and UVB-Induced DNA Damage in Melanomagenesis. *Int. J. Mol. Sci*. 14, 1132-1151, 2013.