BRIGHTENYL®

Contents

1. Introduction .......................................................................................................................... 5
2. Brightenyl®'s Description ..................................................................................................... 5
3. Skin color and skin protection .............................................................................................. 6
4. Skin Color ................................................................................................................................ 6
3.1. Skin protection by melanin ................................................................................................. 6
3.2. Skin protection by melanin ................................................................................................. 6
3.3. Melanin .................................................................................................................................. 6
3.3.1. Melanin synthesis and melanin types .............................................................................. 6
3.3.2. Melanosome transfer ..................................................................................................... 7
5. Brightenyl®’s mode of Action ............................................................................................... 9
6. In vitro and ex vivo assessments of Brightenyl® ................................................................ 10
6.1. Introduction ........................................................................................................................ 10
6.2. Materials and methods ...................................................................................................... 10
6.2.1. Assessment of epidermis penetration of Brightenyl® (THBG) in vivo using Raman microscopy .................................................................................................................. 10
6.2.2. Assessments of Brightenyl® conversion into THBA by skin microbiote .................. 11
6.2.2.1. Microbial cell collection (swabbing) and isolation ...................................................... 11
6.2.2.2. Biotransformation .................................................................................................... 11
6.2.2.3. Microbial growth characterization ............................................................................. 11
6.2.2.4. HPLC-UV analysis of the culture broth during incubation ....................................... 11
6.2.3. Assessments of the presence α-glucosidase genes in skin microbiome using metagenomic and bioinformatic analysis ................................................................................. 11
6.2.3.1. Metagenomic collection ............................................................................................ 12
6.2.3.1.1. Microbial cell collection (swabbing) and isolation .................................................. 12
6.2.3.1.2. DNA extraction and library preparation ................................................................. 12
6.2.3.1.3. Sequencing of the flow cell .................................................................................. 12
6.2.3.1.4. Data processing .................................................................................................... 12
6.2.3.2. Bioinformatic analysis ............................................................................................... 12
6.2.3.2.1. Gene prediction ................................................................................................... 12
6.2.3.2.2. Taxonomic and functional analysis ........................................................................ 12
6.2.4. Assessments of protective effects .................................................................................. 13
6.2.4.1. Antioxidant assay: DPPH assay .............................................................................. 13
6.2.4.2. DNA protectant assay: “Photoprotection” Comet assay ........................................ 13
6.2.5. Assessment of anti-melanogenesis properties ................................................................. 15
6.2.5.1. Anti-melanogenesis studies on pigmented skin explants ........................................... 15
6.2.5.1.1. Description of pigmented skin explants ................................................................. 15
6.2.5.1.2. Brightenyl® treatments of skin explants and culture conditions ........................... 15
6.2.5.1.3. UVA/UVB irradiations ......................................................................................... 15

FM-097B Version 03 / 10.13.2015 2/39
6.2.5.1.4. Histological analysis ................................................................. 15
6.2.5.1.4.1. Preparation of explants for histological analysis ....................... 15
6.2.5.1.4.2. Melanin content visualization (Fontana Masson’s staining) ........ 15
6.2.5.1.4.3. MITF immunostaining and MITF positive cells counting ............ 16
6.2.5.1.4.4. PGE2 immunostaining ......................................................... 16
6.2.5.1.4.5. Image analysis and statistical analysis ..................................... 16
6.2.5.2. Anti-melanosome transfert: Cell lectin interaction study .................. 16
6.2.5.3. Anti-inflammatory assay: NF-κB activity study in transformed human cells 16
6.3. Results and discussion ........................................................................ 17
6.3.1. Brightenyl® vibrational spectra and penetration profile ...................... 17
(Raman study): ......................................................................................... 17
6.3.2. Metagenomic analysis of skin microbiote ......................................... 18
6.3.3. Brightenyl® conversion into THBA by skin microbiote ...................... 19
6.3.4. Protective effects studies: .................................................................. 20
6.3.4.1. Antioxidant activity of Brightenyl® (DPPH assay) ............................ 20
6.3.4.2. DNA Photo-protecting effect of Brightenyl® (Comet assay) .......... 20
6.3.5. Anti-melanogenesis studies (ex-vivo studies results): ......................... 22
6.3.5.1. Melanin visualization and quantification ........................................ 22
6.3.5.2. MITF expression .......................................................................... 22
6.3.5.3. PGE2 expression .......................................................................... 23
6.3.6. Anti-melanogenesis effect studies (in vitro studies results): ............... 23
6.3.6.1. Anti-melanosome transfert: Lectin interaction study ....................... 23
6.3.6.2. Inhibition of NF-κB activity .......................................................... 24
7. Clinical investigation of Brightenyl® ....................................................... 25
7.1. Introduction ......................................................................................... 25
7.2. Materials and methods of clinical tests .................................................. 25
7.2.1. Description of the creams used ......................................................... 25
7.2.2. Description of the panel and study condition ..................................... 25
7.2.3. Chromameter analysis: L*, a* and ITA parameters ............................ 25
7.2.4. Visia- CR analysis ............................................................................ 26
7.2.5. Siascope analysis: Evaluation of melanin content in epidermis .......... 26
7.2.6. Data analysis and Statistical analysis ................................................ 26
7.3. Results and discussion ........................................................................ 26
7.3.1. Chromameter analysis ..................................................................... 26
7.3.1.1. L* parameter ................................................................................ 27
7.3.1.2. a* parameter ............................................................................... 28
7.3.1.3. ITA parameter .............................................................................. 29
7.3.2. Visia-CR analysis ............................................................................. 30
7.3.2.1. UV spots analysis ......................................................................... 30
7.3.2.2. Visia CR pictures ......................................................................... 31
BRIGHTENYL®

7.3.3. Siascope analysis ................................................................. 32
7.3.3.1. Melanin content quantification ........................................ 32
7.3.3.2. Melanin content visualization ......................................... 33
7.4. Conclusions on clinical investigations .................................... 34
8. General Conclusions ............................................................... 36
9. Bibliographic references ....................................................... 37
BRIGHTENYL®

1. Introduction

Brightenyl® also called THBG is an alpha-glucoside derivative of THBA (trihydroxy benzoic acid), a known potent but highly unstable tyrosinase inhibitor. THBG is perfectly stable and highly water-soluble ingredient, which has been developed by Induchem Companies as a safe whitening and skin complexion agent.

Brightenyl® is the very first cosmetic active ingredient, which is activated by a new functional layer of the human skin: the stratum microbium™. This new layer acts as an active living veil converting Brightenyl® in two molecules (Trihydroxybenzoic acid and Trihydroxybenzoic acid glucoside), which act on seven biological targets to obtain a perfect skin complexion.

The present technical report contains the assessment results of Brightenyl® in vitro, ex vivo and in vivo (clinical investigation on human volunteers).

2. Brightenyl® Description

**Identification:**
INCI name (suggested)
WATER, GLYCERIN, DIGLUCOSYL GALLIC ACID

**Appearance:**
yellow liquid

**Solubility:**
Soluble in water

**Safety assessment:**
Ocular irritation: HET CAM; Non-irritant
Skin irritation: Patch test: Non-irritant
Mutagenicity: Ames assay; Non-mutagenic
Sensitization: HRIPT assay; Non-sensitizing

**Dosage:**
1% to 4%

**Storage:**
Recommended storage temperature: 4-8°C
Do not store at temperatures over: 25°C

**Shelf life**
2 years
BRIGHTENYL®

3. Skin color and skin protection

3.1. Skin Color

The diversity of human skin color (white skin to brown/black skin) is a result of a combination of specific skin chromophores[1]. The skin chromophore red is represented by oxyhaemoglobin, blue by deoxygenated haemoglobin, yellow-orange by carotene (an exogenous component), and brown by melanin. As melanin is the major component influencing skin color, natural color or “pigmentation” of skin is dependent on the quantity, type and organization of melanin in the skin (leading to ethnicity variability)[2, 3]. Melanin organization and quantity also determine how the skin will appear after UV exposure, for example during tanning[4]. The more melanin is present, the darker the overall skin tone is and the darker the skin can become upon exposure to UV radiation. UV radiations induce an immediate and delayed tanning in darker skin types[5] and erythema in individuals with light skins. UV induced tanning is due to the increase transfer of melanosomes to keratinocytes and to the increase of melanin production. Skin color is not only dependent on the type of melanin and intensity of melanin in the melanocytes but also on melanosomes characteristics[6] (arrangement, size and number) and melanosomes autophagic process[7]. Melanosomes distributions vary across different skin types. Melanosomes in keratinocytes of light skin as in Caucasians are distributed as membrane-bound clusters, whereas the melanosomes in keratinocytes of dark skin as in African/American individuals tend to be larger and distributed individually[8]. Melanosomes within keratinocytes of Asian skin are distributed as a combination of individual and clustered melanosomes[6].

3.2. Skin protection by melanin

Melanin protects skin from sun-damages as DNA damage, photo-aging and skin cancer[9-11]. DNA damages are greater in fair skins in comparison to darker skins[9, 12]. In the skin melanin and mainly eumelanin provides the natural UV protection by eliminating the generated free radicals/ROS[13].

Melanin pigment provides skin photo-protection, with darker skins having the highest rate of photo-protection[14]. Darker skin type (phototype VI) had a natural intrinsic SPF 10 times that of skin phototype I[15]. As a consequence of this intrinsic SPF, less UV radiation reach dermis in darker skins, so people with darker skin are less sensitive to photo aging.

3.3. Melanin

3.3.1. Melanin synthesis and melanin types

Melanin is a pigment found throughout the human body in skin, eye, brain, hair, and inner ear. Melanin in the skin is produced by melanocytes located in the basal layer of the epidermis under the influence of various endogenous factors, derived from neighboring keratinocytes and underlying fibroblasts[16, 17]. Melanin synthesis is also called melanogenesis and occurs within specialized organelle (melanosomes) where the amino acid L-tyrosine serves as the starting precursor. There are two types of melanin: pheomelanin (yellow to reddish color) and eumelanin (brown to black color). Although these classes share a common biosynthetic origin, specific molecular reactions occurring early in pigment production differentiate these two types[18]. Tyrosinase is the rate-limiting enzyme in melanin biosynthesis. Melanin synthesis is catalysed by at least three enzymatic proteins, tyrosinase (monophenol dihydroxyphenylalanine (DOPA) oxygen oxydo-reductase[19]) and tyrosinase-related proteins (tyrp1 and tyrp2). TRP-2 is also now known as DOPAchrome tautomerase (DCT). Tyrosinase, tyrp1 and tyrp 2 constitute the tyrosinase gene family. Tyrosinase catalyses the rate-limiting generation of L-dopaquinone from L-tyrosine and is also able to oxidize L-dopa to L-dopaquinone (fig 1). Dopaquinone is then cyclized to form dopachrome, which is decarboxylated into 5,6-dihydroxyindole (DHI) or tautomerized to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by tyrp2[20, 21]. DHI and DHICA are then oxidized respectively by tyrosinase and tyrp1[22] to form eumelanin. In the presence of thiols, such as cysteine, dopaquinone binds to cysteine to form 5-S-cysteynlldopa and 2-S
cysteinyldopa. The oxidation of cysteinyldopa isomers in melanocytes leads to the production of pheomelanin (fig 1).

There is no difference in the number of melanocytes between darker or fairer skins but it is the amount and relative ratio of each type of melanin, which contributes in part to the skin’s overall color. The ratio between pheomelanin and eumelanin in fairer skin type melanosomes is increased more markedly than that in melanosomes from dark skin melanocytes[24]. Epidermis melanin and precisely eumelanin content was found to be higher in sun-exposed skin in comparison to non-exposed skin whatever the ethnicity[2, 25]. Differences in skin pigmentation (constitutively or after a stimuli such sun exposure) are a consequence of differences in the activity and expression of tyrosinase, tyrp1 and tyrp2[26]. These 3 enzymes are MITF’s downstreams. MITF means Microphthalmia-associated Transcription Factor and it’s a dimeric transcription factor. MITF is a master regulator of melanogenesis[27, 28] and interacts in the transcriptional regulation of essential regulating proteins involved in melanin production and melanocyte dendricity for example (tyrosinase, tyrp1, tyrp2, Pmel1, Mart1….)[29]. UV-irradiation induced the increased expression of MITF and its downstream melanogenic proteins[30].

3.3.2. Melanosome transfer
Melanosome transfer process is absolutely required for visible pigmentation and effective photo-protection[31-33].
Once produced in the cell body of the epidermal melanocyte, pigmented melanosomes are translocated down the dendrites and captured at the dendritic tips via various cytoskeletal elements. Each melanocyte extends multiple dendrites and contacts a defined group of epithelial cells, creating a pigmentary unit[34]. These melanosomes are then transferred from the dendrites into the epithelial cells. Surface lectins and glycoproteins facilitate this melanosome transfer process[35-37]. The mechanism of transfer is the subject of debate, as epithelial cells have been proposed to acquire...
pigment via: (i) mechanisms based on intercellular bridges, in which melanosomes travel through pores or channels that directly connect the melanocyte’s cytoplasm to the epithelial cell’s cytoplasm, or (ii) mechanisms based on endocytosis, in which epithelial cells engulf pigment-containing material that, depending on the study, consists of dendrite tips, melanocyte filopodia, melanosome-laden vesicles, secreted melanosomes, or the secreted contents of melanosomes.

4. Skin microbiote or stratum microbium™

Our body lives in symbiosis with an incredible number of beneficial microorganisms, which enable us to maintain our health on a daily basis. This micro-flora is called the human microbiome. It accounts for almost ten times as many cells as in the rest of our body, while representing orders of magnitude more genes than are contained in the human genome. The human skin being the largest exposed organ, it is also one of the largest microbial habitats. The human skin microbiome has a known symbiotic population from 100 to more than 10,000,000 cells per cm² depending of the body area[38]. The mutualistic interaction between humans and skin microflora results from long co-evolutionary processes involving the skin as the physical interface of our body with its outside environment. The traditional microbiology-based methods do not offer a full access and understanding to our skin microbiome, as it is estimated that 50 to 90% of existing skin microorganisms cannot be cultivated in a laboratory. A recent high throughput DNA analysis technology (called metagenomics) has enabled to gain a more exhaustive vision of the skin microbiome, and we are just beginning to understand the microorganisms’ complex relationships with each other and our bodies in addition to the cosmetic products we apply to our skin. The skin microbiome is physically located on the top of our skin, in combination with the stratum corneum. Induchem’s R&D center, libragen, is one of the world leader in the metagenomics technology[39]. They have recently analyzed the human skin microbiome by sampling the superficial layers of the skin of volunteers, and sequencing the genomes of their stratum microbium™. The analysis of 40 billions of DNA bases has enabled to discover that our skin microbiome possesses genes of alpha-glucosidases, highly selective enzymes which could be used to activate cosmetic ingredient and increase their efficacy on skin.

Fig 2: Schematic representation of skin microbiome at the skin surface
5. Brightenyl®’s mode of Action

Raman spectroscopy and in vitro tests on human skin microbiote have shown that once applied on skin, Brightenyl® also called THBG is partially converted into THBA by the enzymatic activity of the microbium stratum layer. This process mimics the concept of pharmaceutical prodrugs, which are activated in the human body to become fully functional.

Delivered in situ, THBA and THBG act in synergy on 7 biological targets to regulate and optimize the skin complexion (fig 3):

1. It captures UV induced free radicals (ROS)
2. It prevents UV-induced DNA damages
3. It reduces the expression of PGE2
4. It controls the NF-kB pathway
5. It controls the expression of MITF
6. It saturates keratinocytes receptors for melanosomes
7. It blocks melanin synthesis even under UV conditions

Fig 3: Mechanism of action of Brightenyl®
6. **In vitro and ex vivo assessments of Brightenyl®**

6.1. Introduction

The aims of preliminary *in vitro* and/or ex-vivo assessments of Brightenyl® were the evaluation of its:

- Protective effects as antioxidant, anti-inflammatory agent and DNA protectant agent.
- Potential mechanisms of action involved in the inhibition of melanogenesis process such as action on MITF expression, anti-inflammatory property (inhibition of NF-KB pathway and PGE2 expression), and action on melanosome transfert via the inhibition of specific lectin (sugar receptors) on keratinocytes membrane surfaces.

6.2. Materials and methods

6.2.1. Assessment of epidermis penetration of Brightenyl® (THBG) in vivo using Raman microscopy

Principle: Raman spectroscopy is an alternative non-invasive method to directly determine penetration of molecules in human skin *in vivo* by focusing a laser into the top layers of the skin and recording the scattered Raman signals[40, 41]. The local environment of the molecules influences their vibrational spectra. With this technique, concentration profiles of penetrated actives and skin component can be directly extracted within the first 20µm of the skin. This covers the full stratum corneum, the most important lipophilic barrier layer in terms of penetration.

The quantification analysis is based on the approach that the Raman spectrum of an active is normalized on the absolute signal of keratin. The concentration of active in skin is represented as mmol active per gram of keratin.

Tests substances:

Aqueous solution of Brightenyl® was used at 5%.

Penetration study:

Baseline measurements were taken before application of test substances (blank). 5% diluted Brightenyl® solution was applied on the left and right volar forearm. The application volume was 80 µl on the treated areas, using an area of 4 cm². The tested product was applied with a micropipette and gentle stream of warm air (30 to 40°C) was used while continuously moving the solution’s droplets across the skin area with the tip of the micropipette. Total evaporation took between 8 to 10 min.

Raman measurements were done at defined time points after product application. Time points are given relative to the first contact of the test solution with the skin. Great care was taken to avoid any loss of actives from the skin’s surface. Therefore, the skin’s surface was not wiped with a dry tissue to remove excess product from the skin, as is usually done in Raman penetration studies.

Time points for Raman measurements:
- Before application of test substance
- 2 h after application of test substance to a different test area
- 4h after application of test substance from the same area as 2h after application
- 24h after application of test substance from the same area as 2h after application

No washing or showering of the volar forearm was allowed between the time points, ie 24 hours. All experiments were conducted as three fold replicates. 10 Raman profiles were acquired at each test area and at each time point from the skin’s surface down to a depth of 24 µm.
6.2.2. Assessments of Brightenyl® conversion into THBA by skin microbiote

Bioconversion of Brightenyl® into THBA by microbial cells isolated from skin surface was investigated using HPLC technology. The study method is detailed in the following sections.

6.2.2.1. Microbial cell collection (swabbing) and isolation

9 persons (4 Caucasian females 26 – 51 years old and 5 Caucasian males 26 – 55 years old) without any symptoms of skin disease were subjected to sampling of different parts of their bodies (cheeks, forehead, nares and forearms). Samples were obtained by swabbing a total surface of about 90 to 120 cm²: 4 leaves of sterile gauze of about 5 x 5 cm were stacked up. In a biological safety cabinet, the sterile gauze pile was soaked in sterile 0.8% sodium chloride contained in a Petri dish (about 3 mL). For executing the swabbing technique, people were using safety gloves. For each selected surface, a swab and fresh sterile salt solution were used. The swab was rubbed several times (5 to 10) at each selected surface and then swirled and pressed against the Petri dish. All the suspensions were mixed and the Petri dishes were rinsed with sterile salt solution. The global suspension was filtered using a 40µm filter (Falcon Blue Nylon Cell Strainer, BD BioSciences 352340) and the volume of the resulting suspension was adjusted to 100 mL. The suspension was centrifuged 30 minutes at 10°C (5525 g, BeckmanCoulter™ Allegra® 25R). The supernatant was removed and the microbial biomass was suspended in 2 mL sterile 0.8% sodium chloride. The optical density at 600 nm was measured and a value of 4 was obtained, corresponding to a population of about 2.10^9 cells per mL.

6.2.2.2. Biotransformation

The culture medium used for performing the biotransformation has the following composition: Starch (SIGMA-ALDRICH, S9765, 10 g/L), Yeast Extract 1 g/L (BIOKAR, A1202HA), Meat extract 1 g/L (BD, 211520), Bacto Tryptone 2g/L (BD, 211705). 20 mL of culture broth were introduced into a baffled 100 mL Erlenmeyer flask. After sterilization (121°C, 20 minutes) and cooling, 3 mL of glycerin-free version of Brightenyl® (66 mM) were introduced in the flask. Finally, 0.1 mL of microbial suspension was used for inoculation. Incubation was performed under agitation at 30°C.

6.2.2.3. Microbial growth characterization

Microbial growth was characterized by measuring the optical density at 600 nm.

6.2.2.4. HPLC-UV analysis of the culture broth during incubation

Samples of culture broth were first centrifuged (Eppendorf tube). An aliquote of supernatant was diluted 10 times with a mixture of methanol and water (60/40). The resulting mixture was directly used for analysis by HPLC (Alliance model 2795, Waters) using a photodiode array detector (wavelength range 260-270 nm).

6.2.3. Assessments of the presence α-glucosidase genes in skin microbiome using metagenomic and bioinformatic analysis

α-glucosidase is the enzyme that could hydrolyze the glucosyl moieties of Brightenyl®: the glucoside linkage between glucosyl residues and trihydroxybenzoic acid (THBA) in one hand, the glucoside linkage between 2 glucosyl moieties constituting an oligosaccharide attached to one of the hydroxyl groups of trihydroxybenzoic acid (THBA) in the other hand. α-glucosidase presence in microbial cells isolated from skin surface was investigated using metagenomic technology and bioinformatic analysis (Libragen expertise). The study method is detailed in the following sections.
6.2.3.1. Metagenomic collection

6.2.3.1.1. Microbial cell collection (swabbing) and isolation

2 persons (males 40 – 55 years old) without any symptoms of skin disease were subjected to sampling of their forehead. Samples were obtained by swabbing a defined surface of about 15 cm². The technic used is described in section 6.2.2.1.

6.2.3.1.2. DNA extraction and library preparation

DNA extraction and purification were achieved according to the protocol described by Griffiths et al[42]. Briefly, microbial cells are disrupted using glass beads (FastPrep®-24, MP Biomedicals™ SARL, Illkirch, France) suspended in 1 volume of Cetyl Trimethyl Ammonium Bromide extraction buffer and 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was then extracted and DNA precipitated with ethanol. DNA was finally purified using the Illumina GFX PCR DNA and Gel Band Purification kit (GE Healthcare, 28-9034-70) according to the manufacturer's instructions. 20 ng of metagenomic DNA were used for the library preparation. Metagenomic DNA was fragmented in a Covaris™ M220 instrument (Woburn, MA, USA) to an average size of approximately 250 bp, according to the supplier suggested protocol. Fragmented DNA was used to synthesize indexed sequencing libraries using the TruSeq Nano DNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA) according to manufacturer recommended protocol. Cluster generation was performed on the cBOT instrument using the TruSeq PE Cluster Kit v3 reagents (Illumina). In short, library fragments are bound to a flow cell by hybridizing the fragments to a lawn of nucleotides complementary to the adapter sequences. Bound fragments are clonally amplified by bridge amplification to create millions of individual dense clusters of clones.

6.2.3.1.3. Sequencing of the flow cell

Libraries were sequenced with an Illumina HiSeq 2000 using the TruSeq SBS Kit v3 reagents (Illumina) for paired end sequencing with reads lengths of 150 base pairs (300 cycles).

6.2.3.1.4. Data processing

Primary analysis (image analysis and base calling) were performed using HiSeq Control Software (HCS) and Real Time Analysis (RTA). Secondary analysis (demultiplexing) was performed using Illumina CASAVA Software (version v1.8). High throughput sequencing reads were quality filtered using the fastq_quality_filter program provided with the FASTX-Toolkit. Only those reads with a quality score higher than 17 for at least 80% of the read length (i.e., probability of correct base call close to 98%) were retained.

6.2.3.2. Bioinformatic analysis

6.2.3.2.1. Gene prediction

Gene catalogs for each sample were created using the MOCAT pipeline[43]. Briefly, the pipeline performs quality control of the raw reads, removes human contamination by mapping to the reference human genome, assemblies the reads and predicts protein-coding genes on the assembled overlapping reads (contigs) and scafflgs (contigs that were extended and linked using the paired-end information of sequencing reads).

6.2.3.2.2. Taxonomic and functional analysis

Predicted proteins were compared to the non-redundant NCBI RefSeq database using BLAST[44]. Taxonomic analysis was performed based on the NCBI taxonomy and functional analysis was performed by MEGAN4 using the SEED classification [45-47]. Taxonomic analysis is performed by placing each sequence read onto a node of the NCBI taxonomy, based on gene content. For each read that matches the sequence of some gene, the program places the read on to the lowest common ancestor (LCA) node of those species in the taxonomy that are known to have that gene. This is called the LCA algorithm.
BRIGHTENYL®

6.2.4. Assessments of protective effects

6.2.4.1. Antioxidant assay: DPPH assay

Principle: The antioxidant activities of molecules were evaluated by determining their abilities to chemically reduce the stable free radical DPPH. Antioxidant activities were evaluated by the spectrophotometric method, using the DPPH radical.

DPPH assay procedure: Brightenyl® (THBG: Trihydroxybenzoic acid glucoside) and its non glucosylated form (trihydroxybenzoic acid THBA) were prepared in ethanol. N-acetyl cysteine (NAC, Sigma-Aldrich®, Saint Quentin Fallavier, France) and Vitamin C (Sigma-Aldrich®), were used as a positive control and prepared in ethanol. Compounds were diluted to a specific range of concentrations (between 0 to 400 μM, depending on the component) in 96-well microplates (100 μl), the control being the solvent alone. A 50 mM stock solution of α,α-diphenyl-β-picrylhydrazyl (DPPH, Sigma) was prepared by dissolving 19.7 mg DPPH in 10 ml of ethanol or methanol, and then a 300 μM intermediate solution was added. Then, 100 μl of DPPH solution (final concentration 150 μM) was added to each well. The plate was shaken to ensure thorough mixing before being wrapped with aluminum foil and placed into the dark. After 15 min, the optical density (OD) was read at the wavelength of 540nm using a microplate reader (Victor 3V, Perkin Elmer, Courtaboeuf, France). All tests were run at least in triplicate and averaged.

Data analysis: The inhibitory effect of various molecules was calculated as a percentage of the control using the following formula: % inhibition = [(1-OD (DPPH + sample)/OD (DPPH alone))] x 100%. EC50 (half effective concentration) was defined as the concentration of a compound decreasing the absorbance of a DPPH solution by 50 %.

6.2.4.2. DNA protectant assay: “Photoprotection” Comet assay

Principle: The Comet assay or Single Cell Gel Electrophoresis assay (SCGE) is an electrophoretic technique on microgel. It allows to detect and to quantify single strand breaks (SSB) and double strand breaks (DSB) of DNA, alkali-labile sites and incomplete repair sites in individual eukaryotic cells exposed to various genotoxic agents including solar irradiation.

In the cell, the DNA is present as a supercoiled double helix. In alkaline conditions, the supercoiled structure unwraps, the DNA denatures itself in single strands and the fragments of DNA are freed. After electrophoresis and treatments, the DNA of an intact cell appears under the shape of a sphere and in cells bearing DNA damages, the DNA takes the shape of a comet. Solar irradiations are responsible of direct and indirect DNA single strand breaks through oxidative stress and cellular repair systems intervention, respectively. “Photoprotection” Comet assay consists in the quantification of the photoprotective property of active ingredients toward the inhibition of SSB formation induced by UVA/visible or broadspectrum irradiation.

Concentration of BRIGHTENYL® tested: 0.2%, 0.4% and 2%

Cells used: Human primary melanocytes were used.

Irradiation procedure: The light source used was a solar simulator SUNTEST CPS+ (XENOTECH, Atlas Material Testing Technology BV, Moussy-le-Neuf, France). The irradiator was equipped with a 1500 watt air cooled Xenon Arc lamp and special glass filters restricting transmission of light below 290 nm or below 320 nm and near IR-blocking filter. The irradiance was fixed at 750 W/m2 throughout the experiments and the combined light dose was 4.5 J/cm² for 1 min irradiation, corresponding to 0.28 J/cm² for UVA, 0.08 J/cm² for UVB and 4.14J/cm² for visible light for broad-spectrum irradiation. This irradiation dose corresponds to 1-3 min period of solar exposure during a clear summer day in the United Kingdom[48]. Cells embedded in agarose on comet slides were irradiated at 4°C on a bed of ice and the comet assay was performed immediately after the irradiation.

Comet assay procedure:

Cells were dispensed into 35 mm diameter tissue culture dishes or 6-well plates (1x10⁵ cells/dish or
well). After cell recovery and adherence, cells were pre-incubated with Brightenyl®, reference compound and buffers at the determined concentrations for 24h (37°C, 5% CO2) or with Methyl methanesulfonate (MMS) 60 µM for 2h.

Then, the alkaline Comet assay was performed as described previously[49-51]. The assay was carried out under yellow light to prevent any additional damage that could be induced by natural light. Briefly, cells was harvested, centrifugated and resuspended in 75 µl of a solution of 0.5% low melting point agarose (LMP) at 37 °C. Cells suspension (5x104) were placed on microscope slides previously coated with 1.8 % agarose and layered with 85 µl of solidified 0.8% agarose gel. After application of a third layer of LMP agarose, slides were immersed in cold lysis solution during 90 min at 4 °C. The microscope slides were then placed in an electrophoresis tank, and DNA was allowed to unwind in freshly prepared alkaline electrophoresis buffer for 20 min at room temperature. Electrophoresis was conducted for 20 min at 25 V and 300 mA. The slides were then washed three times with a cold neutralizing buffer for 5 min and dehydrated in pure methanol.

**Data analysis**

Each slide was stained with 50 µl of 2 µg/ml ethidium bromide and covered with a coverslip. The slides were analyzed with a fluorescence microscope equipped with an oil immersion Apo/Dplan 40Å~ lens connected to a high sensitivity EMCCD LUCA-S camera. The final magnification was 400X. A total of 50 randomly selected cells were analyzed per slide using Komet 6.0 image analysis software (Andor Technology, Belfast, UK). DNA damage was expressed as the Olive Tail Moment (OTM; arbitrary units); 100 OTM values were determined for each sample, 50 from each of two separate slides/sample. Comparisons of means were performed with the SigmaPlot (version 11.0, Systat Software, Chicago, IL, USA). The 0.05 level of probability was used as the criterion for significance.

For the Comet assay, the 100 calculated OTM values/sample were distributed into 40 classes between the minimal and the maximal values. A nonlinear regression analysis was performed on the OTM distribution frequencies by using a χ2 function with Table Curve 2D software (version 5.01; Systat Software, Chicago, IL, USA). The calculated degrees of freedom (n) for this function was quantitative measures of the DNA damage for a sample [Bauer et al., 1998; Jean et al., 2001]. The n was termed χ2 OTM and used as the sole parameter for assessing levels of DNA damage. The significances of the differences between χ2 OTM values of control and treated cells was analyzed using Student's t-test analysis.

The % protections will be determined as follows:

$$\% \text{ Protection} = \left[ 1 - \frac{\chi^2_{\text{OTM}_{\text{PUV}}} - \chi^2_{\text{OTM}_{\text{Co}}}}{\chi^2_{\text{OTM}_{\text{UV}}} - \chi^2_{\text{OTM}_{\text{Co}}}} \right] \times 100$$

With:
- χ2 OTM<sub>Co</sub>: χ2 OTM of the cells without any treatment.
- χ2 OTM<sub>PUV</sub>: χ2 OTM of the cells pre-treated with ingredients and irradiated.
- χ2 OTM<sub>UV</sub>: χ2 OTM of the cells irradiated.
6.2.5. Assessment of anti-melanogenesis properties

6.2.5.1. Anti-melanogenesis studies on pigmented skin explants

Brightenyl® was assessed during extensive ex vivo studies to determine its potential effect as whitening agent. The potential whitening activities of Brightenyl® at different concentrations were explored on pigmented human skin explants maintained in survival. The biological parameters studied were involved in melanogenesis process: Melanin quantity, MITF expression and PGE2 expression. The general morphology of the skin treated with Brightenyl® at each concentration was also observed in comparison to known benchmark (Kojic acid).

6.2.5.1.1. Description of pigmented skin explants

Pigmented skin explant with a phototype III to IV from 43-year-old Caucasian woman was obtained from an abdomino-plasty. 24 explants of an average diameter of 10 mm were prepared. The explants were kept in a survival BEM culture medium (Bio-EC's Explant Medium) at 37°C in a humid, 5%-CO₂ atmosphere.

6.2.5.1.2. Brightenyl® treatments of skin explants and culture conditions

Brightenyl® (without any formulation ingredients except water as a solvent) was topically applied at 1.4mM on the basis of 2 mg per cm², using a small spatula. Explants were treated with Brightenyl® on D0, D1, D2, D3, D5, D7, D8 and D9. The culture medium was refreshed (1 ml) on D1, D3, D5, D7 and D9. On the explants from the batches exposed to UV, products were applied 30 minutes before irradiation.

4 control explants were prepared:
- Samples 1: untreated explants (no active ingredient and no irradiation treatment)
- Samples 2: explants only UV irradiated (no active ingredient)
- Samples 3: explants only treated with Brightenyl® (no UV irradiation)
- Samples 4: explants treated with Kojic acid at 70.4 mM (no UV irradiation)

6.2.5.1.3. UVA/UVB irradiations

On D0, D1, D2, D3, D5, D7, D8 and D9 explants from batches exposed to UV were irradiated at the dose of 1.125J/cm² UVA, (6-8%of UVB) using a Vilbert Lourmat UV simulator RMX 3W. Before irradiation, explants were put in HBSS medium. Un-irradiated batches were kept in dark. After irradiation, all the explants were put in BEM.

6.2.5.1.4. Histological analysis

6.2.5.1.4.1. Preparation of explants for histological analysis

On D0, the 3 explants of the batch D0 were collected and cut in two parts. One half was fixed in buffered formol solution, and the other half was frozen at -80°C.

On D10, 3 explants from each batch were collected and processed in the same way. After fixation for 24 hours in buffered formol solution, the samples were dehydrated and impregnated in paraffin using a Leica TP 1010 dehydration automat. The samples were then embedded using a Leica EG 1160 embedding station. 5-µm-thick sections were made using a Leica RM 2125 Minot-type microtome, and the sections were then mounted on Superfrost® Plus silanized glass slides.

The frozen samples were cut into 7-µm-thick sections using a Leica CM 3050 cryostat. The microscopical observations were realized using a Leica DMLB or Olympus BX43 microscope. Pictures were digitized with an Olympus DP72 camera and the Cell®D data storing software.

6.2.5.1.4.2. Melanin content visualization (Fontana Masson’s staining)

Melanin visualisation has been realized by silver impregnation according to Fontana Masson's staining.
6.2.5.1.4.3. MITF immunostaining and MITF positive cells counting

The immunostaining of MITF was performed on paraffinized sections with a mouse polyclonal anti-MITF antibody (Abcam, ref. Ab-49387, clone 34CA5), diluted at 1:20, for 1 hour at room temperature. The staining was enhanced with a streptavidin/peroxidase system and revealed using VIP system (Vector, SK-4600). The immunostaining was assessed by microscopical observations. MITF positive cells were counted along the epidermis of each explant and expressed per cm of epidermis. Epidermal length was calculated using a Cell^D software.

6.2.5.1.4.4. PGE2 immunostaining

The immunostaining of PGE2 was performed on frozen sections with a rabbit polyclonal anti-PGE2 antibody (Abcam, ref. ab2318), diluted at 1:50, for 1 hour at room temperature. The staining was revealed using AlexaFluor 488 (Lifetechnologies, A11008). The nuclei were post-stained with propidium iodide. The immunostaining was performed using an automated slide-processing system (Dako, AutostainerPlus). The immunostaining was assessed by microscopical observation.

6.2.5.1.4.5. Image analysis and statistical analysis

The images analyses were realized on 8 to 10 images for each batch using Cell^D software. Statistical analysis of the data was performed using “t-test” of student for paired data. The statistic significances are reported as follows: n.s. not significant p>0.05 and * significant p<0.05.

6.2.5.2. Anti-melanosome transfert: Cell lectin interaction study

Concentrations of Brightenyl® tested: 0.14%, 0.4%, 2 and 4%. Neoglycoprotein used: α-Galactose-BSA fluoresceinylated. αGalactose-BSA fluoresceinylated were synthetized, labeled and carrefully controled by the supplier. Assay procedure: Normal human primary keratinocytes, were cultured on 96 wells plates, and incubated with fluoresceinylated neoglycoproteins alone or mixed with a range of concentrations of Brightenyl® for 4h at 4°C. After washing, fluorescence was recorded and corrected according to intrinsic fluorescence of neoglycoprotein.

6.2.5.3. Anti-inflammatory assay: NF-κB activity study in transformed human cells

Principle of Assay of NF-κB activity: The contact of IL1-beta and transfected cells induced gene expression of the NF-κB pathway as well as SEAP (secreted Embryonic Alkaline Phosphatase) loaded by the plasmid pNiFty2-SEAP. The expression of this gene and the production of the SEAP enzyme were directly proportional to the quantity of cytokine present in the culture media. The assay of SEAP enzyme is an indicator of NF-κB pathway activation. Concentration of Brightenyl® tested: 0.4%, 0.8%, 2% and to 4% Cells cultures and treatments: Transformed human cells (CaCo-2/C17) were transfected with a NF-κB reporter gene plasmid (pNiFty2-SEAP, reference: pNifty2-SEAP, Invivogen, Toulouse, France). The transfected cells (50000/well) were incubated 24 h with IL-1 beta (10 ng/mL) in the presence or absence of Brightenyl® at different concentrations. All experimental conditions were performed in triplicate. Assay procedure: The assay of the enzyme SEAP was performed using the Quantie blue reactif (ref rep-qb, Invivogen, France) according to the instructions of use provided by the fabricant. The enzyme concentration was determined by the reading of the absorbance at 655 nm.

Data analysis: Raw data were analyzed with Microsoft Excel software. All reported data are expressed as mean ± sem. The standard error of the mean (sem) is calculated as the standard deviation (sd) divided by the square root of sample size. Standard error of the mean: sem = Sd/√n.

A percentage of inhibition was determined by the comparison of the mean values of control condition and treated conditions:

\[ \% \text{ Inhibition} = 100 - \left( \frac{\text{mean value of treated condition}}{\text{mean value of untreated condition}} \right) \times 100. \]
6.3. Results and discussion

6.3.1. Brightenyl® vibrational spectra and penetration profile
(Raman study):
Vibrational spectrum of Brightenyl® is shown in fig 4.

Fig 4: Calibrated Raman spectrum of Brightenyl®.

Since the product present at the skin surface was not removed by wiping, there was still some sample remaining on the surface of the skin (fig 5B).

Fig 5: Micrograph (600x) of untreated (A) and Brightenyl® treated skin surface right after evaporation of the solvent (B) as seen through the Raman microscope. Remaining Brightenyl® is clearly seen on the surface of the skin as oily areas showing interference patterns (black arrows). The black cross-indicate the position of the excitation laser (switched off).

Brightenyl® epidermis penetration after 2 hours was demonstrated as seen by the relative product concentration calculated at 8 and 12µm in comparison to Brightenyl® concentration found at the surface (table 1):

<table>
<thead>
<tr>
<th>Depth of epidermis (8µm)</th>
<th>Relative Brightenyl® concentration in comparison to the surface at (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At 8µm</td>
<td>17,4 ± 0,21</td>
</tr>
<tr>
<td>At 12µm</td>
<td>7,1 ± 0,28</td>
</tr>
</tbody>
</table>

Table 1: Relative Brightenyl® concentration in the epidermis (Raman spectroscopy)
A maximum of product quantity was observed in the stratum corneum after 2 h, while fewer product quantity was observed after 4 h and no product was observed at 24h revealing a total product penetration 24h after product application (fig 6).

Traces of THBA were detected during the experiment (data not shown). THBA penetrates into the skin up to a maximum limit of 8 µm (data not shown).

### 6.3.2. Metagenomic analysis of skin microbiote

Metagenomic analysis of the 24 samples of skin microbiote leads to the generation of 270,154,762 sequences of 150 bp. This metagenomic analysis corresponds to the analysis of 40,523,214,300 DNA bases. Such a comparison this number of DNA bases analyzed is equivalent to 15, 666 times to S epidermidis genome (average size: 2,5 Mbp). This analysis leads to the identification of 394,070 genes. From these genes, 122 were identified as alpha glucosidase. 20 species were associated to these 120 alpha glucosidase genes:

- 11 *Propionibacterium acnes* species (acnes, sp. KPL2008, SK187, HL025PA1, HL050PA1 HL082PA2, HL103PA1, HL202PA1, JCM 18916 and JCM 18918)
- 4 *staphylococcus species* (capitis, epidermidis, sp. AL1 and warneri)
- *Micrococcus luteus*
- *Macrococcus caseolyticus*
- *Kytococcus sedentarius*
- *Kocuria palustris*
- *Dermacoccus sp. Ellin185*
6.3.3. Brightenyl® conversion into THBA by skin microbiote

As shown in table 2 at T0, Brightenyl® is a solution of trihydroxybenzoic acid glucosides (mono called “THBG” and polyglucosides called “other glucosides THBA”) and traces of trihydroxybenzoic acid called “THBA”. We have shown in this study that the glucosylated trihydroxybenzoic acid derivatives (THBG) are transformed into THBA by the microbial cells isolated from skin samples. Indeed, as seen in table 2 there is a significant increase of THBA after 72 hours of Brightenyl® incubation with skin microbiote. This demonstrates the release of THBA from THBG and other glucosides TBHA. In the same time, it is clearly showed that THBG decreases after incubation period of 24, 48 and 72h and there is no more TBHA after 96 and 163 hours. The increase of TBHA concentration at 72 hours table 2 shows clearly that the microbial cells are able to catalyze the hydrolyzis of the glucosidic linkage between TBHA and glucose.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Incubation times Brightenyl® with skin microbiote</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detected</td>
<td>T0</td>
</tr>
<tr>
<td>components</td>
<td></td>
</tr>
<tr>
<td>by HPLC-UV</td>
<td></td>
</tr>
<tr>
<td>THBA</td>
<td>++</td>
</tr>
<tr>
<td>THBG</td>
<td>+++</td>
</tr>
<tr>
<td>Other glucosides</td>
<td>+++</td>
</tr>
</tbody>
</table>

Table 2: HPLC-UV analysis of the culture broth after different incubation times of Brightenyl® and skin microbiote (0, 24, 48,72, 96 and 163h)

In the same time, the concentration of other glucosides THBA decrease, demonstrating that the microbial cells are able to catalyze the hydrolysis of glucosidic linkages between several glucose residues. THBA component initially found in Brightenyl® is also probably used by the skin microbiote or oxidized as seen by its concentration decrease from 0 to 48 hours (no more TBHA in the culture broth was detected, table 2).

Conclusions on Raman and skin microbiote studies (conversion study and metagenomic analysis)

• Using Raman technology, we have found after application of Brightenyl® on skin surface some traces of THBA.
• Using metagenomic analysis, we have found that several species of skin microbiote get alpha glucosidase gene.
• During the THBG conversion study in presence of skin microbiote, we confirmed that Brightenyl® is converted into THBA.
  ➢ All these results confirm that stratum microbium™ has alpha glucosidase activity, which can convert THBG into THBA.
**BRIGHTENYL®**

6.3.4. Protective effects studies:

6.3.4.1. Antioxidant activity of Brightenyl® (DPPH assay)

Both products THBG (Brightenyl®) and THBA got intrinsic antioxidant property as seen by the concentration dependent increasing percentage of DPPH reduction (figure 7). The anti-radical scavenging activity is stronger with THBA than THBG. Both products were more effective in DPPH reduction than the controls N-acetyl cysteine and Vitamin C (Figure 7 and table 3). The anti-oxidant power of THBA is 5 times more important than Brightenyl® as demonstrated by EC50 values and 4 more important than vitamin C. The anti-oxidant power of Brightenyl® is comparable to Vitamin C.

![Graph](image)

**Fig 7**: Effect of THBG (glucosylated form of THBA), THBA (non glucosylated molecule) versus vitamin C and N-acetyl cysteine (NAC) on DPPH reduction. NAC and Vitamin C were used as positive controls for anti-oxidant properties. Effective concentrations EC50 have been determined as the concentration leading to 50% of the maximal DPPH reduction.

<table>
<thead>
<tr>
<th>Product</th>
<th>EC 50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THBG (Brightenyl®)</td>
<td>87</td>
</tr>
<tr>
<td>THBA</td>
<td>17</td>
</tr>
<tr>
<td>N acetyl cysteine</td>
<td>137</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>74</td>
</tr>
</tbody>
</table>

Table 3: Effective concentrations EC50 for THBG, THBA, N acetyl cysteine and vitamin C determined from DPPH reduction experiments.

6.3.4.2. DNA Photo-protecting effect of Brightenyl® (Comet assay)

In this assay, we have confirmed that broad-spectrum irradiation induced direct DNA breaks (table 4). The DNA breaks are a consequence of ROS production induced by broad spectrum irradiation. The Comet assay was validated by MMS treatment showing an increased in DNA single strand breaks. Pre-incubation with Brightenyl® at all tested concentrations resulted in significant (**p inf 0.001) lower levels of DNA single strand breaks after irradiation close to the levels of the non irradiated condition (control). Brightenyl® induced a dose-response relationship in protection of cells against DNA lesions induced by broad-spectrum irradiation in normal human melanocytes. The level of broad-spectrum photoprotection ranged from 72.5% to 94.1%.
Treatment with increasing concentrations of Brightenyl® has shown an increasing photoprotectant effect (table 4 and fig 8).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>OTM</th>
<th>X2 OTM</th>
<th>Protection %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.27 ± 0.02</td>
<td>2.09 ± 0.08</td>
<td>100</td>
</tr>
<tr>
<td>MMS (60 µM)</td>
<td>52.88 ± 0.25</td>
<td>14.61 ± 0.54***</td>
<td>NA</td>
</tr>
<tr>
<td>Broad spectrum irradiation (4.5 J/cm²)</td>
<td>47.55 ± 0.26</td>
<td>12.93 ± 0.39***</td>
<td>0</td>
</tr>
<tr>
<td>Brightenyl® 0.2% + irradiation</td>
<td>19.12 ± 0.14</td>
<td>5.07 ± 0.22***</td>
<td>72.5</td>
</tr>
<tr>
<td>Brightenyl® 0.4% + irradiation</td>
<td>11.24 ± 0.08</td>
<td>3.94 ± 0.10***</td>
<td>82.9</td>
</tr>
<tr>
<td>Brightenyl® 2% + irradiation</td>
<td>7.43 ± 0.07</td>
<td>2.73 ± 0.17***</td>
<td>94.1</td>
</tr>
</tbody>
</table>

Table 4: Photoprotective properties of Brightenyl® at 0.2%, 0.4% and 2% against broad spectrum damages in normal human melanocytes. Calculated protection % is also included. ***: P< 0.001

Figure 8 illustrates different typical comets that were detected for control cells and irradiated cells with Brightenyl® treatment.

Fig 8: Pictures of typical comets obtained from melanocytes treated by different conditions. Brightenyl protect melanocyte DNA as seen by the absence of DNA damage tail.

Conclusions on protective effect studies

Based on *in vitro* studies, we have shown that:

- Brightenyl® is a potent antioxidant active ingredient with antioxidant property up to 4 times better than vitamin C and 8 times than N acetyl cysteine.
- Brightenyl® is a potent DNA photoprotectant as demonstrated by Comet assay on normal human melanocyte UV irradiated: +94% of photoprotectant effect.
6.3.5. Anti-melanogenesis studies (ex-vivo studies results):

6.3.5.1. Melanin visualization and quantification

On non irradiated samples (fig 9):
After 10 days of treatment and comparatively to the blank batch (BD10), the melanin content in melanocyte and in keratinocyte in basal layer is decreased in samples treated with Brightenyl® at 1.4mM by 31%. Brightenyl® induces a whitening activity (-31%), better than the reference kojic acid at 1% corresponding to 70.4mM (-22%) (fig 9). The effect seen is concentration dependent (data not shown).

![Fig 9: Melanin visualization on pigmented human skin explants treated with Brightenyl® at 1.4 mM during 10 days. Kojic acid at 1% (70.4 mM) was used as a reference. (Histological staining: Fontana masson staining)](image)

On irradiated samples (fig 10):
After 10 days of treatment and comparatively to the blank batch (BDUV10), the melanin content in melanocyte and in keratinocyte in basal layer is decreased in samples treated with Brightenyl® at 1.4mM by -16% (fig 10).

![Fig 10: Melanin visualization on pigmented human skin explants treated with Brightenyl® at 1.4mM and UV irradiated during 10 days. Human skin explants only UV irradiated were used as control. (Histological staining: Fontana masson staining)](image)

6.3.5.2. MITF expression

On irradiated samples (table 5):
After 10 days of culture and compared to untreated explants (no active ingredient and no irradiation), we noticed that UV irradiation induces an increase of MITF positive cells by 28%.
After 10 days of Brightenyl® treatment at 1.4mM and UV irradiation, we noticed that the number of MITF positive cells is decrease in comparison to samples UV irradiated. This decrease was concentration dependent with respectively -5% and -37% of MITF positive cells (data not shown).
**Table 5:** MITF positive cells counted in pigmented human skin explants treated with Brightenyl® at 2 and 4% and UV irradiated during 10 days. Human skin explants only UV irradiated were used as control. (Immunostaining)

**6.3.5.3. PGE2 expression**

On irradiated samples (fig 11):
After 10 days of culture, an increase of PGE2 expression in comparison to day 0 was noticed in the untreated explants.
After 10 days of culture in comparison to the untreated explants, an increase of PGE2 expression was seen after UV irradiation.
After 10 days of Brightenyl® treatment at 1.4mM and UV irradiation, a decrease of PGE2 was seen.

Fig 11: PGE2 expression in pigmented human skin explants treated with Brightenyl® at 1.4mM and UV irradiated during 10 days. Human skin explants only UV irradiated were used as control. (Immunostaining)

**6.3.6. Anti-melanogenesis effect studies (in vitro studies results):**

**6.3.6.1. Anti-melanosome transfert: Lectin interaction study**

The results have shown that Brightenyl® induced a strong inhibition, concentration dependent, of the interaction between galactose receptor and galactose neoglycoproteins. These results suggested that Brightenyl® interact strongly with galactose receptor expressed at the surface of keratinocytes (fig 12).

Figure 12: Inhibition of neoglycoprotein interactions with galactose receptor on normal human keratinocyte by Brightenyl®

(cell lectin interaction study)
6.3.6.2. Inhibition of NF-κB activity

Treatment with increasing concentrations of Brightenyl® inhibited up to 90% of the NF-κB activation pathway activity induced by IL-1 beta in human cells (fig 13).

![Figure 13: Inhibition of NF-κB activity by Brightenyl®](image)

**Conclusions on anti-melanogenesis effect studies**

Based on ex vivo studies, we have shown that:

- Brightenyl® at 1.4mM (4%) has shown a whitening effect on irradiated and non-irradiated pigmented skin explants after 10 days of treatment.

- Brightenyl® effect is better than kojic acid as demonstrated by quantification of melanin content. A significant decrease of melanin content by -31% at 1.4 mM was seen in comparison to -22% for kojic acid at 70.4 mM, showing a 60 times better dose/ratio activity of Brightenyl® vs Kojic acid.

Based on in vitro and ex vivo studies, we have shown that the mechanism of action of Brightenyl® as a whitening and anti-redness agent is due to the:

- Decrease of melanosome transfer by its physical interaction with galactose receptors.

- Decrease of MITF positive cells number (-37% under 1.4 mM of Brightenyl® treatment) by decreasing the expression of MITF expression at the protein level.

- Anti-inflammatory properties:
  - Inhibition of NFκB activation pathway up to -90% inhibition at 1.4 mM (4%)
  - Decrease of PGE2 expression
7. Clinical investigation of Brightenyl®

7.1. Introduction

Based on the promising results obtained in vitro and ex vivo demonstrating that Brightenyl® is able to decrease the melanogenesis process, we decided to investigate this anti-melanogenesis and skin tone regulating activity in a double-blinded clinical study involving human Korean volunteers having face spots.

Due to the fact that skin color is not only due to melanin synthesis but also to skin redness we focused also our investigation on the global analysis of skin color: global pigmentation (ITA parameter), redness (a* parameter) and brightness (L* parameter).

To evaluate the:

- effect of Brightenyl® on melanin synthesis seen ex-vivo, the epidermis melanin content was assessed by the use of complementary tools (histological analysis and siascope analysis).
- photoprotective effect of Brightenyl® seen in vitro, we analyzed UV spots by Visia-CR tool. (Nota: UV spots happen when melanin coagulates below the skin's surface because of sun exposure. Indeed reducing UV spots epidermis content enables to decrease the level of future hyperpigmented spots at the skin's surface).

7.2. Materials and methods of clinical tests

7.2.1. Description of the creams used

Cream compositions (INCI composition):
AQUA, ALCOHOL DENAT, GLYCERIN, GLYCERYL STEARATE SE, SIMMONDSIA CHINENSIS SEED OIL, CETEARYL ALCOHOL, PALMITIC ACID, STEARIC ACID, TOCOPHEROL, HELIANTHUS ANNUUS SEED OIL, PERFUME +/- 2 % Brightenyl®.

7.2.2. Description of the panel and study condition

A double blind and placebo-controlled clinical evaluation was carried out with 20 Korean women (age between 30 and 60 years old, mean age: 46 ± 7 years) showing clinical signs (face spots). All of the subjects participating in the study gave their informed consent signed at the beginning of the study.

Mean ITA scores of skin volunteers faces were 33,28 ± 8 on non-spot area and 21 ± 8 on spot area.

The effect of Brightenyl® at 2% on skin pigmentation was evaluated at days 15, 30, 60 and 84 days after daily product use. The volunteers applied twice a day either a placebo cream on one side of their face or a cream containing 2% Brightenyl®. Dermatologists assessed the following face skin parameters: skin color (L*, a* and ITA parameters using chromameter tool) and melanin content (Visia and siascope tools).

7.2.3. Chromameter analysis: L*, a* and ITA parameters

The measure of the skin colour intensity was conducted by colorimetric method performed with a chromameter (Chromatique CM 2600dTM MINOLTA, France). Chromameter is a portable spectrophotometer with a measurement sensor surface of 1cm². The sensor is applied on the skin surface for 3 seconds. The surface can be decreased for measure on a dark spot.

The parameters L*, a*, b* and Chroma C* were recorded:

\[ \text{Chroma C*} = [(a*)^2 + (b*)^2]^{1/2} \]

The CIE L*a*b* system is a classification allowing to define an absolute colour by determining its position in a 3 dimensional space:
**BRIGHTENYL®**

Vertical coordinate L* defines lightness from zero (black) to 100 (maximum lightness).
The position in each horizontal plan, abscissa (from − a* = green to + a* = red) and ordinate (from − b* = blue to + b* = yellow) defines its chroma.

Melanin absorbs all the wavelengths from visible spectra so it globally darkens the color of the skin. Therefore ITA (Individual typological angle) parameter was used for measuring the intensity of pigmentation of the skin. ITA index was calculated as follows: arctang ((L* - 50)/b*)*(180/π).

Three repeated measures were carried out on the dark spot on each volunteer, at all time-points. Only the average is taken into account.

Constitutively dark skin types have lower L* values, higher a* values and higher b* values than constitutively light skin types[52]. Dark skin types have also lower ITA value and light skin type have higher ITA value[14]. If the ITA increases then the quantity of melanin decreases.

7.2.4. Visia- CR analysis

Using the VISIA-CR, digital facial pictures were taken at each study time (D0, D14, D28, D56 and D84). Visia CR was used to analyze UV spots. The results analyzed by VISIA-CR were retrieved as absolute scores.

UV spots happen when melanin coagulates below the surface of the skin because of the sun exposure. UV spots are generally invisible under normal lighting. Visia detects stains through the selective absorption of UV light by the epidermal melanin.

Visia CR calculated one measure for each parameter, on each volunteer for each product tested, at all study time. Only the average was taken into account.

7.2.5. Siascope analysis: Evaluation of melanin content in epidermis

Using the Siascope, the melanin content was quantified at the dark spot area. The results were expressed in arbitrary units. The SIAscope V is a digital epiluminescence microscopy system designed for taking shadow-free magnified images of skin. It is generally used to measure and image the amount of melanin present in the epidermal layer of skin. The instrument consists of a handheld probe containing light sources and a digital imaging sensor. 2 measures were carried out on the dark spot on each volunteer, at all time point. Only the average was taken into account.

7.2.6. Data analysis and Statistical analysis

The mean and standard deviation were calculated for each parameter measured at all time point. Then, the calculation of percentage variation compared to the initial measurement allowed the assessment of the overall effect of the product studied in comparison to placebo. Statistical analysis of the data was performed using “ t-test” of student for paired data. The statistic significances are reported as follows: n.s. not significant p>0.05 and * significant p<0.05.

7.3. Results and discussion

7.3.1. Chromameter analysis

Assessment of the skin color evolution after Brightenyl® and placebo treatments were conducted using a chromameter tool. The chromameter converts colors into a digital code:

- L* for clarity (from dark to light). If the L* parameter is high, the skin color is light.
- a* for the green to red spectrum. If a* parameter increased, the redness of skin increased.

The individual typological angle (ITA) which defines the degree of skin pigmentation was also determined. The higher ITA parameter is, the lighter the skin pigmentation is.
**BRIGHTENYL®**

7.3.1.1. **L* parameter**

Brightenyl® used at 2% induced a significant increase of L* parameter from D28 to D84 demonstrating a lightening effect on the skin (fig 14 and table 6). The rate of improvement varies from +0.9% to +2.2%. The improvement is time dependent, indeed L* parameter increased from D14 to D84. No significant improvement was seen with the placebo.

![Graph showing L* parameter changes](image)

*Fig 14: L* parameters measured at D0 and after 14, 28, 56 and 84 days of placebo and Brightenyl® treatments (chromameter tool, n=20 volunteers, each measurement was done in triplicate, # and ** represent the significant effect seen respectively with p<0.1 and p<0.01, student t test, paired data).*

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Placebo</th>
<th>Brightenyl® at 2%</th>
<th>Performance average variation of Brightenyl® vs placebo (%) (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L* (Mean ± standard deviation) (Arbitrary unit)</td>
<td>Average variation vs D0 (%) (P value)</td>
<td>L* (Mean ± standard deviation) (Arbitrary unit)</td>
</tr>
<tr>
<td>D0</td>
<td>57.31± 3.4</td>
<td>-0.14% (p=0.601)</td>
<td>57.16+ 2.10</td>
</tr>
<tr>
<td>D14</td>
<td>57.16 + 3.01</td>
<td>-0.14% (p=0.601)</td>
<td>57.27 + 1.85</td>
</tr>
<tr>
<td>D28</td>
<td>57.40+ 3.54</td>
<td>0.05% (p=0.794)</td>
<td>57.71 + 1.95</td>
</tr>
<tr>
<td>D56</td>
<td>57.67 + 3.55</td>
<td>0.36% (p=0.145)</td>
<td>58.16 + 2.22</td>
</tr>
<tr>
<td>D84</td>
<td>57.63 + 3.7</td>
<td>0.32% (p=0.278)</td>
<td>58.40 + 2.21</td>
</tr>
</tbody>
</table>

*Table 6: L* parameters measured at D0 and after 14, 28, 56 and 84 days of placebo and Brightenyl® treatments (chromameter tool, n=20 volunteers, each measurement was done in triplicate, # and ** represent the significant effect seen respectively with p<0.1 and p<0.01, ns for non significant, student t test, paired data).*
7.3.1.2. **a* parameter**

Brightenyl® used at 2% induced a significant decrease of a* parameter after 28 days of use. The effect seen was maintained after 56 and 84 of daily use demonstrating an anti-redness effect on the skin. No significant improvement was seen with the placebo. In comparison to placebo Brightenyl® used at 2% induced a significant decrease of a* value by -600% after 84 days of treatment (fig 15 and table 7).

![Graph showing a* parameters](image)

**Fig 15**: a* parameters measured at D0 and after 14, 28, 56 and 84 days of placebo and Brightenyl® treatments (chromameter tool, n=20 volunteers, each measurement was done in triplicate, # and * represent the significant effect seen respectively with p<0.1 and p<0.05, student t test, paired data).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Placebo</th>
<th>Brightenyl® at 2%</th>
<th>Performance average variation of Brightenyl® vs placebo (%) (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a* (Mean ± standard deviation) (Arbitrary unit)</td>
<td>Average variation vs D0 (%) (P value)</td>
<td>a* (Mean ± standard deviation) (Arbitrary unit)</td>
</tr>
<tr>
<td>D0</td>
<td>11.75 ± 2.01</td>
<td>-1% (p=0.7148)</td>
<td>12.67 ± 2.13</td>
</tr>
<tr>
<td>D14</td>
<td>11.63 ± 2.51</td>
<td>-3% (p=0.1059)</td>
<td>12.14 ± 2.57</td>
</tr>
<tr>
<td>D28</td>
<td>11.23 ± 2.14</td>
<td>-3% (p=0.3173)</td>
<td>11.65 ± 2.21</td>
</tr>
<tr>
<td>D56</td>
<td>11.45 ± 2.09</td>
<td>-3% (p=0.3173)</td>
<td>11.77 ± 1.96</td>
</tr>
<tr>
<td>D84</td>
<td>11.64 ± 32.74</td>
<td>-1% (p=0.7639)</td>
<td>11.78 ± 2.71</td>
</tr>
</tbody>
</table>

**Table 7**: a* parameters measured at D0 and after 14, 28, 56 and 84 days of placebo and Brightenyl® treatments (chromameter tools, n=20 volunteers, each measurement was done in triplicate, # and * represent the significant effect seen respectively with p<0.1 and p<0.05, ns for non significant, student t test, paired data).
7.3.1.3. ITA parameter

Brightenyl® used at 2% induced a significant increase of ITA parameter about 10.4% and 11.5% respectively after 56 and 84 days of use demonstrating a lightening effect on the skin. The improvement seen with Brightenyl® is time dependent (ITA parameter increase from D14 to D84). No improvement was seen with the placebo. In comparison to placebo and after 84 days of use Brightenyl® induced a significant increase of ITA parameter by 16 times (fig 16 and table 8).

Fig 16: ITA parameters calculated at D0 and after 14, 28, 56 and 84 days of placebo and Brightenyl® treatments (chromameter tool, n=20 volunteers, each measurement was done in triplicate, * and ** represent the significant effect seen respectively with p<0.05 and p<0.01, student t test, paired data).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Placebo</th>
<th>Brightenyl® at 2%</th>
<th>Performance average variation of Brightenyl® vs placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ITA (Mean ± standard deviation) (Arbitrary unit)</td>
<td>Average variation vs D0 (%) (P value)</td>
<td>ITA (Mean ± standard deviation) (Arbitrary unit)</td>
</tr>
<tr>
<td>D0</td>
<td>20.92 + 10.11</td>
<td>-2.74% (p=0.42)</td>
<td>20.82 + 6.42</td>
</tr>
<tr>
<td>D14</td>
<td>20.34 + 8.44</td>
<td>-1.95% (p=0.34)</td>
<td>21.00 + 5.43</td>
</tr>
<tr>
<td>D28</td>
<td>20.64 + 9.47</td>
<td>-3.3% (p=0.34)</td>
<td>21.67 + 5.68</td>
</tr>
<tr>
<td>D56</td>
<td>21.61 + 10.02</td>
<td>-3.29% (p=0.30)</td>
<td>22.99 + 5.80</td>
</tr>
<tr>
<td>D84</td>
<td>21.60 + 10.10</td>
<td>-3.29% (p=0.30)</td>
<td>23.20 + 5.24</td>
</tr>
</tbody>
</table>

Table 8: ITA parameters calculated at D0 and after 14, 28, 56 and 84 days of placebo and Brightenyl® treatments (chromameter tools, n=20 volunteers, each measurement was done in triplicate, * and ** represent the significant effect seen respectively with p< 0.1; p<0.05 and p<0.01, student t test, paired data).
7.3.2. Visia-CR analysis

Although both the chromameter and the VISIA-CR use L*a*b* technology to measure skin color changes, the sensitivity of the VISIA-CR to measure skin color changes is higher when compared to the Chromameter[53]. VISIA-CR is a non-invasive tool allowing visualization of full face skin area while providing inherent algorithms for analysis of specific skin parameters such as brown spots and UV spots.

7.3.2.1. UV spots analysis

Brightenyl® used at 2% induced a significant decrease about 7% of UV spots after 28, 56 and 84 days of daily use. After 28 and 84 days of placebo treatment use a significant increase in UV spots was seen from 6.8 to 9.3%. In comparison to placebo, Brightenyl® induced a significant decrease of UV spots by a value up to 18 times after 84 days (figure 18 and table 10).

Fig 18: UV spots parameters quantification (#, * and ** represent the significant effect seen respectively with p <0.1; p<0.05 and p<0.01, student t test, paired data, Visia CR Tool analysis).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Placebo</th>
<th>Brightenyl® at 2%</th>
<th>Performance average variation of Brightenyl® vs placebo (in times) (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV spots (Mean ± standard deviation) (Arbitrary unit)</td>
<td>UV spots (Mean ± standard deviation) (Arbitrary unit)</td>
<td>UV spots (Mean ± standard deviation) (Arbitrary unit)</td>
<td>Performance average variation of Brightenyl® vs placebo (in times) (p value)</td>
</tr>
<tr>
<td>D0</td>
<td>0.213 ± 0.060</td>
<td>0.209 ± 0.070</td>
<td></td>
</tr>
<tr>
<td>D14</td>
<td>0.212 ± 0.063</td>
<td>-0.4% (p=0.8825)</td>
<td>0.203 ± 0.064</td>
</tr>
<tr>
<td>D28</td>
<td>0.228 ± 0.074</td>
<td>6.8% (p=0.0809)</td>
<td>0.193 ± 0.060</td>
</tr>
<tr>
<td>D56</td>
<td>0.226 ± 0.065</td>
<td>3.3% (p=0.521)</td>
<td>0.194 ± 0.058</td>
</tr>
<tr>
<td>D84</td>
<td>0.233 ± 0.075</td>
<td>9.3% (p=0.0188)</td>
<td>0.194 ± 0.064</td>
</tr>
</tbody>
</table>

Table 10: UV spots parameters quantification (#, * and ** represent the significant effect seen respectively with p <0.1; p<0.05 and p<0.01, student t test, paired data, Visia CR Tool analysis).
7.3.2.2.  Visia CR pictures

Pictures taken using Visia CR under cross-polarized light confirmed the whitening effect of Brightenyl® used at 2% (fig 19). Indeed Brightenyl® brighten the skin color of full hemi face treated and help to even skin tone. A decrease of skin pigmentation was also noticed in hyperpigmented areas as seen in the pictures below by white circles. The skin tone is also more homogenous.

Fig 19: Pictures taken using before and after 84 days of Brightenyl® treatments. Circles represent hyper-pigmented areas (Visia CR tool with cross polarized light).
7.3.3. Siascope analysis

7.3.3.1. Melanin content quantification

Brightenyl® used at 2% induced a significant decrease from 4% to 7.5% from 14 to 84 days of daily use. The placebo treatment induced also a significant decrease in melanin content by about 3% from D28 to D84. The effect seen was better and stronger with Brightenyl® treatment than with placebo. In comparison to placebo a significant decrease of melanin content by +150 % was quantified after 84 days of Brightenyl® treatment (fig 20 and table 11).

![Graph showing melanin content quantification](image)

**Table 11:** Melanin content quantification at D0 and after 14, 28, 56 and 84 days of placebo and Brightenyl® treatments (Sciascope tool, n=20 volunteers, each measurement was done in triplicate, * and ** represent the significant effect seen respectively with p<0.05 and p<0.01, student t test, paired data).
7.3.3.2. Melanin content visualization

Using siascope tool specific melanin content pictures were obtained. The results have shown as illustrated by the volunteer n°2, that the active ingredients Brightenyl® has an anti-melanogenesis property. On the pictures (fig 21) we notice a more pronounced decrease in melanin content on the spot area and the non-spot area treated with Brightenyl® at 2% in comparison to placebo. These results were consistent with melanin quantification reported in section 6.3.3.1.

Fig 21: Melanin content visualization at D0 and after 14, 28, 56 and 84 days of placebo and Brightenyl® treatments (Sciascope tool, selection of 1 volunteer). Melanin content is not decrease in placebo side in comparison to Brightenyl treatment where a clearly decrease of melanin content is seen in spot area (represented by dark spot “black color”) and in normal area represented by grey color.
7.4. Conclusions on clinical investigations

The clinical investigation has shown that the cream containing Brightenyl® at 2% significantly improves all clinical parameters studied (L* "Luminosity", a* "Redness", ITA "Brightness", UV spots and melanin content) after 28, 56 and 84 days of use. The effect is time dependent as confirmed by the significant difference seen between Brightenyl® and placebo treatment.

**After 28 days of treatment:**

<table>
<thead>
<tr>
<th>Parameters at D28</th>
<th>Brightenyl 2%</th>
<th>Placebo</th>
<th>Difference seen between Brightenyl® and placebo at the statistically level (yes or no) and the percentage of improvement calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>L* (Luminosity)</td>
<td>+0.9%</td>
<td>No effect</td>
<td>No</td>
</tr>
<tr>
<td>a* (Redness)</td>
<td>-8%</td>
<td>No effect</td>
<td>No</td>
</tr>
<tr>
<td>UV spots</td>
<td>-7.9%</td>
<td>+6.8%; Increase UV spots</td>
<td>Yes/ Improvement by 16 times</td>
</tr>
<tr>
<td>Melanin content</td>
<td>-5.3%</td>
<td>-4.3%</td>
<td>No</td>
</tr>
</tbody>
</table>

**After 56 days of treatment:**

<table>
<thead>
<tr>
<th>Parameters at D56</th>
<th>Brightenyl 2%</th>
<th>Placebo</th>
<th>Difference seen between Brightenyl® and placebo at the statistically level (yes or no) and the percentage of improvement calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>L* (Luminosity)</td>
<td>+1.8%</td>
<td>No effect</td>
<td>Yes</td>
</tr>
<tr>
<td>a* (Redness)</td>
<td>-7%</td>
<td>No effect</td>
<td>Yes/ Improvement by -167%</td>
</tr>
<tr>
<td>ITA (Brightness)</td>
<td>+10.4%</td>
<td>-3.3%</td>
<td>Skin pigmentation increase (Result non statistically-significant)</td>
</tr>
<tr>
<td>UV spots</td>
<td>-7.4%</td>
<td>+3.3%; Increase UV spots (Result non statistically-significant)</td>
<td>Yes Improvement by 11 times</td>
</tr>
<tr>
<td>Melanin content</td>
<td>-5.1%</td>
<td>-3 %</td>
<td>No</td>
</tr>
</tbody>
</table>
After 84 days of treatment:

<table>
<thead>
<tr>
<th>Parameters at D84</th>
<th>Brightenyl 2%</th>
<th>Placebo</th>
<th>Difference seen between Brightenyl® and placebo at the statistically level (yes or no) and the percentage of improvement calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>$L^*$(Luminosity)</td>
<td>+2.2%</td>
<td>No effect</td>
<td>Yes</td>
</tr>
<tr>
<td>$a^*$(Redness)</td>
<td>-7%</td>
<td>No effect</td>
<td>Yes/ Improvement by -600%</td>
</tr>
<tr>
<td>ITA (Brightness)</td>
<td>+11.5%</td>
<td>-3.3%; Skin pigmentation increase (Result non statistically-significant)</td>
<td>Yes/ Improvement by 16 times</td>
</tr>
<tr>
<td>UV spots</td>
<td>-7.5%</td>
<td>+9.3%; Increase UV spots</td>
<td>Yes/ Improvement by 18 times</td>
</tr>
<tr>
<td>Melanin content</td>
<td>-7.5%</td>
<td>-3%</td>
<td>Yes/ Improvement by -150%</td>
</tr>
</tbody>
</table>

Based on these clinical parameters, it appears clearly that Brightenyl® is a potent brightening and skin complexion improving agent. Brightenyl® mode of action is due to the modulation of skin colors (red component and brown component). Indeed, Brightenyl® is able to decrease the red component of the skin as seen by the decrease of $a^*$ parameter. Brightenyl® also targets the brown component of the skin color as confirmed by increase of ITA parameter and decrease of melanin content which is directly correlated with skin pigmentation. Brightenyl® is a multifunctional skin color and skin tone and skin complexion optimizing agent.
8. General Conclusions
Brightenyl® is a cosmetic ingredient activated by the newly discovered layer of the human skin: the startum microbium™. This activation has been demonstrated by means of raman spectroscopy and exhaustive metagenomic analysis of the human skin microbiote.

We have shown that Brightenyl® is a potent whitening agent with comparable or better results than kojic acid (based on human pigmented explant analysis).

The mode of action discovered for Brightenyl® by in vitro studies rely on:

- its antioxidant properties (comparable to vitamin C)
- its anti-inflammatory properties (inhibition of NFKB pathway and inhibition of PGE2 synthesis pathway)
- its DNA photoprotectant effect (Comet assay)
- its anti-melanogenic effect as seen by the interaction with specific lectins involved in melanosome transfert and decreased MITF expression at the protein level.

This ingredient acts simultaneously on 7 biological targets.

- it captures UV induced free radicals (ROS)
- it prevents UV-induced DNA damages
- it reduces the expression of PGE2
- it controls the Nf-kB pathway
- it controls the expression of MITF
- it saturates keratinocytes receptors for melanosomes
- it blocks melanin synthesis even under UV conditions

We have demonstrated on Asian volunteers, the time dependent efficient effect of Brightenyl® (from 28 days to 84 days of use).

- Luminosity and brightness increase (L* and ITA parameters increase)
- Redness decrease (a* parameter decrease).
- Decrease melanin content of the skin, which is also confirmed, by the decrease of UV spots.

All clinical parameters assessed were significantly improved after 84 days of treatment in comparison to placebo.

Brightenyl® is a perfectly safe active ingredient: no irritation no side effects have been noticed by the dermatologist during the clinical assessment.

Brightenyl® is a multifunctional active ingredient with several customers benefits:
1. Photo protection
2. Powerful antioxidant (4x more potent than vitamin C)
3. Redness correction
4. Brightness increase (60x better than kojic acid)
5. Age spot removal
6. Skin tone uniformization
9. Bibliographic references

BRIGHTENYL®