LIGHTOCEANE®

Potent skin lightener from the Ocean

*

Influences all main pathways involved in melanogenesis

Complementary anti-aging & radicals scavenging activities

Gelyma

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SUMMARY

INTRODUCTION

Skin colour results from the presence of chromatophores in the skin (Pierard, G.E., 1998 – J. Eur. Acad. Dermatol.Vener. 10: 1-11). It is mainly determined by the nature and quantity of natural pigment, melanin, present in the epidermal layers of skin.

Pigmentary disorders are seen frequently. They are related from various factors, such as photo-induced ageing, hormonal disorders (contraception, pregnancy, menopause …) repeated sun exposure and inflammation reactions. As a result, the skin complexion becomes not uniform.

For Asian populations, light skin is considered as a symbol of beauty and youth.

So, skin pigmentation becomes an international concern.

The actual cosmetic market trends attempt to satisfy an increased demand for a more radiant and uniform complexion by developing novel actives used:

- either to lighten the skin complexion,
- or to adjust difference in pigmentation and to eliminate “age spots”,

with increased efficacy and improved safety profiles.

Moreover, skin lightening is seen as a part of the anti-ageing skin care.

GELYMA has developed a concept based on innovation, effectiveness and tolerance with the help of *in vitro* and *ex vivo* studies.

This novel active ingredient LIGHTOCEANE® is obtained from the brown alga *Halydris siliquosa*, never used previously for cosmetic purposes to our knowledge.

LIGHTOCEANE® is effective with supporting data for skin lightening activity by targeting diverse steps occurring before, during and after melanin synthesis.

- Before melanin synthesis, LIGHTOCEANE® reduces the release of nitric oxide that is an important melanogenic mediator.

- During melanin synthesis, LIGHTOCEANE® inhibits the activity of tyrosinase and the synthesis of melanin.

- After melanin synthesis, LIGHTOCEANE® controls the transfert of melanosomes to keratinocytes by inhibiting the dendricity of melanocytes.

With these complementary potentialities at several levels of the melanogenesis cascade, LIGHTOCEANE® can successfully down-regulate skin pigmentation.

LIGHTOCEANE® also shows preventive protection by neutralising free radicals and protecting against UV radiations, both being known as major triggers of melanogenesis.

LIGHTOCEANE® prevents inflammation by inhibiting both lipoxygenase and interleukine IL1α, as many skin-lightening actives which are under current use, have irritating potential on the skin.

As IL1α is suspected to be involved in the formation of age spots LIGHTOCEANE® may be useful for the prevention of age spots.

LIGHTOCEANE® produces as well the capacity to boost skin cells and to inhibit elastase and collagenase activities against dermal deterioration.

All this make LIGHTOCEANE® attractive as a novel and innovative active ingredient from natural and marine origin.

LIGHTOCEANE® offers a clever mechanism of action for an optimal and safe lightening effect efficacy, associated to anti-irritation and anti-ageing properties.

LIGHTOCEANE® is safe, non mutagenic and non promutagenic. The RIPT test on 105 volunteers has proved that it does not in any way cause irritation or elicit an allergic response.

Gelyma Patent: FR 2 942 136.

THE MECHANISM OF MELANOGENSIS

Melanogenesis is a complex enzymatic process which transforms tyrosine into eu- and pheomelanin, responsible for skin pigmentation.

It is related to the presence of specialized cells: melanocytes.

In the human epidermis, melanocytes originate from the neural crests from which melanoblasts migrate to the basal layer of the epidermis where they differentiate into mature melanocytes having the complete machinery to produce melanin. They have dendritic extensions which allow them to come into contact to several keratinocytes.

Melanocytes represent approximately 5% of the cells of the basal area of the epidermis. They have one main function: melanin synthesis or melanogenesis.

Melanin is synthetized and stored in specialized intracellular organelles termed melanosomes which contain several enzymes that mediate the production of melanin.

Melanin is transported from the center of the cell to the peripheral dendrite tips where they may be transferred to the surrounding keratinocytes to produce skin pigmentation.

Among melanin-synthesizing enzymes, tyrosinase is the most important. It catalyses the first two steps of melanin synthesis:

- hydroxylation of tyrosine giving 3,4 dihydroxyphenylalanine (DOPA)

- oxidation of DOPA to DOPAquinone.

Once the reactive DOPAquinone is produced, DOPAchrome is spontaneously formed.

Further oxidation and reduction reactions involve the action of several regulatory factors *e.g.* tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2). They ultimately convert DOPAchrome to melanins.

Melanogenesis is mainly stimulated by UV radiation which acts according to different effects on melanocyte proliferation (see Ichihashi M*. et al*., 2003 – IFSCC magazine 6(4) 279-286). Some of them are direct, others are indirect.

The direct effects of UV radiation imply:

- modification of membrane phospholipids (Park H.Y. *et al.,* 1999 – J. Biol. Chem. 16470-16478).

- DNA damage and DNA repair (Gilchrest B.A. *et al*., 1993 – J. Invest. Dermatol. 101: 666-672).

- action of nitric oxide (NO) (Romero-Graillet C*. et al.* 1996 – J. Biol. Chem . 28052-28056).

The indirect effects of UV radiation on melanogenesis are induced through different stimulators produced in and released from the neighbouring keratinocytes *e.g.*:

- α-melanocyte stimulating hormone (α MSH) (Hunt G.*et al*., 1994- J.Endocrinol. 140: R1-R3)

- endotheline-1 (ET-1) (Imokawa G. *et al*, 1995 – J. Invest. Dermatol., 105 : 32-37)

- several inflammatory factors (Tomita Y. *et al*., 1992 – Pigment Cell Research 5: 357-361; Morelli J.G. & Norris DA. , 1993 – J. Invest. Dermatol. 100: 191S-195S,).

So, the melanization of epidermis is a dynamic event involving many factors.

Each person contains both types of melanins. However, the perceived colour of skin is determined by the ratio of eumelanins to pheomelanins.

Generally melanin formation takes place harmoniously in the skin. However, pigmentary disturbances can occur, especially hypermelanosis is frequent. It linked to the increased activity of the melanocytes or melanocyte proliferation.

Pigmentary disorders alter appearance. The psychological and socio-economic impacts of skin pigmentation and depigmentation are great in Humans.

In Asian countries, pigmented spots are one of the major problems to be solved.

Consequently, interest in discovery of new skin-lightening agents is currently on the increase by cosmetic customers.

From the pathways of melanin synthesis, skin-lightening can be achieved:

-by targeting the inhibition of the tyrosinase activity or

-by blocking the chain reaction at the various points of the pathways (see Jimbow K. & Minamitsuji, Y., 2001- Dermatol. Ther. 14: 35-45; Briganti S. *et al*., 2003 –Pigment Cell Res. 16 : 101-110).

LIGHTOCEANE® is able to inhibit this chain reaction by focusing diverse steps occurring before, during and after melanin synthesis.

ALGAL SOURCE

LIGHTOCEANE® is extracted from the brown seaweed: *Halidrys siliquosa*.

➤ Classification

The species *Halidrys siliquosa* belongs to:

Empire	*Eukaryota*
Kingdom	*Chromista*
Infrakingdom	*Heterokonta*
Phylum	*Ochrophyta*
Class	*Phaeophyceae*
Order	Fucales
Family	*Sargassaceae*
Genus	*Halidrys* Lyngbye 1819
Species	*siliquosa* (Linnaeus) Lyngbye 1819.

• Synonyms

Fucus siliquosus Linnaeus, 1753
Cystoseira siliquosa (Linnaeus) C.Agardh 1820.

• Origins of the name

From Greek "*hals*" : the sea and "*drus*" : an oak
From Latin "*siliquose*"

• Common names

Sea oak — Great Britain

Rupán
Schotentang
Pod-weed
Meereiche
Crůba préacháin
Fraoch freangach
} Ireland

➤ Morphology & Biology

The brown alga *Halidrys siliquosa* can reach 120 cm in length, occasionally up to 200 cm (Fig.1). Young plants are olive-green in colour while older thalli are dark brown and leathery.

Fronds are linear, compressed, distichously pinnate or bi-pinnate. They rise from a strong, flattened cone shaped holdfast.

The main axe is flattened with branches, for the most part, disposed in one plane.

These branches arise alternatively from the margins of their respective parent axes. They appear usually slightly flattened and give a distinctly zigzag appearance.

Certain laterals develop into characteristic and mucronate air-bladders. These air-vesicles are linear-lanceolate and stalked (about 0.5 cm wide by 1-4 cm long). They resemble pods or siliquae whence the specific name (Fig.2).

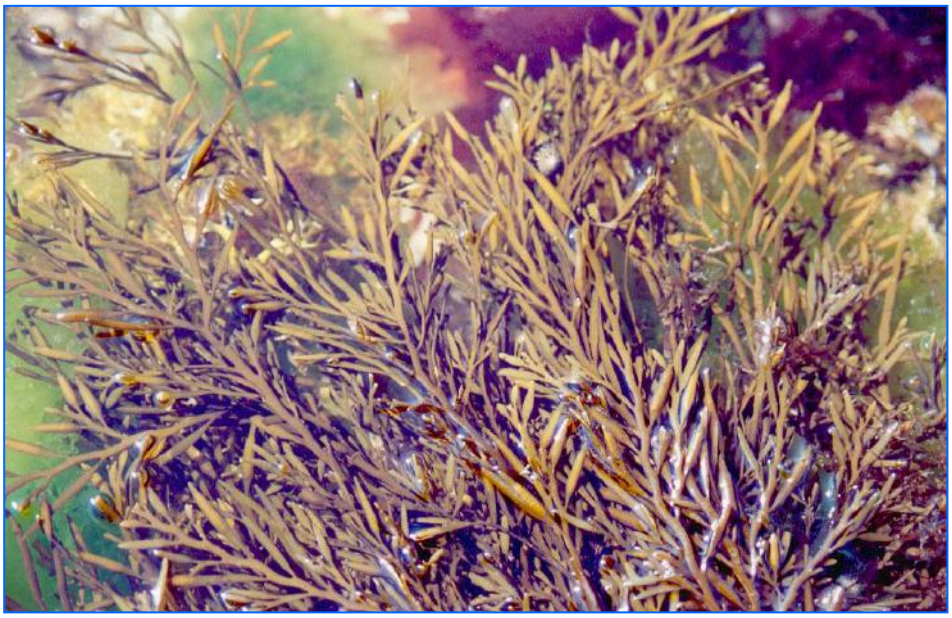


Fig. 1– Morphology of *Halidrys siliquosa*

Fig.2- Aspect of air-vesicles

Air-vesicles are differentiated during the second year's growth (Moss, B. & Lacey, A., 1962 – New Phytologist , 62 : 67-74).

The adult thallus shows a seasonal periodicity in growth and reproduction. There is a period of rapid vegetative growth during the spring and early summer which is followed by receptacle differentiation.

Receptacles are located at the apices of the branches. They bear spherical conceptacles which contain both antheridia and oogonia.

Gametes are formed shortly before liberation from the receptacles. They discharge from December to March. (Moss, B. & Lacey, A., 1962 – New Phytologist, 62 : 67-74).

Zygotes are large and may form clusters that sink rapidly. They are covered by adhesive mucus and stick to the substratum (Hardy, F.G. & Moss, B., 1978 –Phycologia, 17:69-78).

This alga shows high protection against epiphytes. It regularly cast off its "skin", the outer layers of their outermost cell walls (Moss, B., 1982 – Phycologia 21:185-191), as a result it is often free of epiphytes.

➤ Ecology & Geographical distribution

The species *Halidrys siliquosa* is marine and perennial with a life span of at least three years (Moss, B. & Lacey , A ., 1962 – New Phytologist , 62 : 67-74).

It grows permanently submerged. It has a wide vertical distribution from pools at high water spring tides to below the sublittoral fringe.

The species *Halidrys siliquosa* is European Atlantic endemic.

It is a common species from the north of the Scandinavia Peninsula to Brittany, in north-west France. (*cf.* Luning, K., 1990 – Fig. 2).

It may be only collected along the Western coasts of the Baltic sea (Pankow,H., 1971 – Algenflora der Ostsee, 419p.).

It is completely absent on the French Basque coasts. Its presence has been recorded along the Galician coasts (Margalet, J.L. & Navarro, M.J., 1990- Botanica Complutensis, 16: 99-107) and recently along the Portuguese coasts (Lima , F.P. *et al.,* 2008 – JMBA2 – Biodiversity Records).

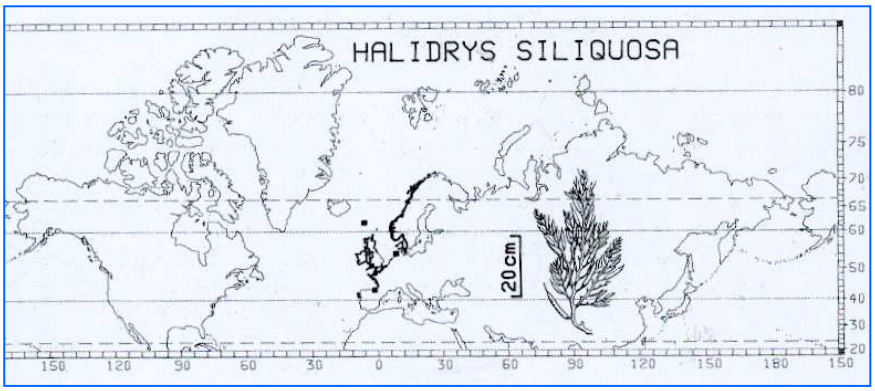


Fig. 3– Geographical distribution of *Halidrys siliquosa*

cf. K. Lüning, 1990 - Seaweeds, their environment, biogeography and ecophysiology, Wiley, NY.

➤ Chemical composition

The amount of total minerals present in *Halidrys siliquosa* reaches 11.19 % (dry mass) (Vinogradov, A.P., 1953 – The elementary chemical composition of Marine organisms, Moscow, 647p).

Cell walls contains about 16-17 % of alginic acid (dry mass) (*cf.* Chapman, V.J. & Chapman , D.J. , 1980 – Seaweeds and their uses , 334 p. Chapman & Hall, London, N-Y).

The ratio mannuronic acid / guluronic acid varies from 1.1 to 0.75 (*cf.* in Stewart, W.D.P. – 1974 - Algal Physiology & Biochemistry, Blackwell Scientific Publications, Ltd.).

Are also present cellulose about 14 % (dry mass) and a water soluble polysaccharide fraction about 62 % (Cronshaw J. *et al*., 1958 – Biochim & Biophys. Acta , 27: 89-103).

The total proteins content equals 9.6 % (dry mass), the most abundant free amino acids being aspartic acid, threonine and alanine (Citharel, J. 1971 – Thesis, Rennes).

The total lipids content reaches 3.61 % (dry mass), glycolipids being the most common. The total fatty acids content has been evaluated to 4.0 $mg.g^{-1}$ (dry mass) (Fleurence, J. *& al.,* 1994 – J. Applied Phycol. 6:527-532).

Biotin has been detected (0.21 µg/g in May) by Larsen, B. (1961 – Norsk. Inst.for tan-og taref, Report 26).

The amount of phenols varies from 5.6 to 15.4 % mg-g^{-1} (dry mass) according to the season, the period of harvest ect… (*cf.* Haug, A. & Larsen, B. 1958 – Norsk. Inst.for tan-og taref, Report 22, 18p); Glombitza, K.W. & Sattler, E. 1973 – Tetrahedron Letters. 43: 4277-4280).

Free phloroglucinol has been detected (Glombitza, K.W. *& al.,* 1973 – Planta Med. 24: 301-303).

Numerous oligomers of phloroglucinol can be identified as phlorotannins : fucols (difucol) and fuhalols (bi-, tri-, tetra-, penta- and heptafuhalols) (*cf*. Glombitza ,K.W., 1979 in Marine Algae in Pharmaceutical Science, eds H.A. Hoppe *& al*., : 303-342 , Walter de Gruyter, Berlin, N.Y.).

The polyphenolics compounds of brown algae differ from the tannins derived from terrestrial plants by their biosynthesis pathway.

Other secondary metabolites have been isolated too *e.g.* meroditerpenoids (tetraprenyltoluquinol-related metabolites) and several hydroquinols (Culioli G. *& al.*, 2008 – J. Natural Products, 71(7) : 1121-1126); Higgs , M.D. & Mulheiron, L.J., 1981 – Tetrahedron, 37 (18) : 3209-3213).

➤ Bioactivities & Utilizations

The bioactivities are linked to the special chemical composition of this brown seaweed.

The cellulose of *Halidrys siliquosa* may be used as a nicotine carrier in pharmaceutical compositions such as tablet, transdermal patch or medicated chewing gum. This cellulose has a high capacity of binding nicotine and reversibly releases it in its gaseous form (Pfizer Health, AZB, Patent WO/2005/023227).

Some extracts of *Halidrys siliquosa* give positive haemagglutins reactions (Blunden G. *et al*., 1978 in Irvine, D.E.G. *et al.,* – Modern Approaches to the Taxonomy of Red and Brown Algae, Academic Press, London, U.K).

The antibiotics activities of *Halidrys* extracts would be due to the presence of :

- acetylated phenols (Sattler, E. *& al*., 1977 – Tetrahedron, 33(10) : 1239-1244).

- acrylic acid (Chesters, G.C.C. & Stott, J.A., 1956 – Proc. 2nd Int. Seaweed Symp. 49-53 ; Hornsey, I.S. & Hide, D., 1974 – Br. Phycol. J., 9 : 353-361).

In brown algae, the low-molecular weight polyphenols appear to have antifungal, antiviral and antibiotic properties.

The antifouling activities of *Halidrys siliquosa* would be linked to the presence of meroditerpenoids (Culioli G. *et al.*, 2008 – J. Natural Products, 71(7): 1121-1126).

Such antibiotic and antifouling activities may be related to novel applications to control the growth of algae in paints and to antifouling paints (Bayer, A.G., Patent WO9851154).

Halidrys siliquosa can be also a source of alginate (Chapman, V.J. & Chapman, D.J., 1980 – Seaweeds and their uses, 334 p. Chapman & Hall, London, N-Y).

THE ACTIVE INGREDIENT LIGHTOCEANE®

Specifications

on a control batch

- appearance : liquid limpid brown coloured with possible brown precipitates
- odour : typical
- pH : 5 ± 1
- density : 1.02 ± 0.02
- dry residual (%) : 3.6 ± 0.4
- solubility : soluble in ethanol, propylene glycol, butylene glycol
  : insoluble in oils.

- microbiology : bacteria : < 100 germs / ml.
  : yeasts, moulds : < 100 germs / ml.
  : pathogens : free.

Composition

Ingredients		Amounts %
Solvent	water	52
Brown alga	*Halidrys siliquosa* extract	48
Preservative	as required	
Others (antioxidants …)	none	

INCI names: water CAS n° 7232-18-5 EINECS n°: 231-791-2.
Halidrys siliquosa extract.

See other INCI versions of LIGHTOCEANE® page 41

♦ Contents in minerals

The contents in minerals have been evaluated by the testing Company EUROFINS (France).

➢ Macrominerals (ppm)

Potassium : 2100
Sodium : 1600
Magnesium : 220
Calcium : 120

➢ Trace minerals (ppm)

Iron : 220
Silicon : 24
Zinc : 0.9
Manganese : 0.35
Selenium : < 0.1
Copper : < 0.1

Iodine : 13.4

➢ Heavy metals (ppm)

Arsenic	: 0.1
Cadmium	: < 0.1
Mercury	: < 0.1
Lead	: < 0.1

♦ Content in polyphenols

The total content of polyphenolic compounds has been evaluated by the Folin-Ciocalteu assay. The principle of the method is based on the oxidation of phenols in the presence of phosphomobybdic and phosphotungstic acids leading to the formation of blue compounds, the absorption maximum of which being at 760 nm. The calibration series is prepared from phloroglucinol.

LIGHTOCEANE® contains 0.16 % total polyphenols phloroglucinol equivalent.

Storage

LIGHTOCEANE® should be stored in the original sealed drums, under clean conditions between 15 to 25°C. In order to avoid microbial secondary contamination, it is recommended to use the whole content of the drum once opened.

If stored under the recommended conditions, LIGHTOCEANE® remains stable for at least 18 months.

Pack size: 1 Kg – 5Kg – 10 Kg.

Safety

No animal experimentation.

Standard safety testing proves that LIGHTOCEANE® is safe for cosmetic use at the recommended use levels:

- slightly irritant for ocular irritation (Het Cam test)

- no irritant for dermal irritation (Human Patch test)

- no mutagenic and no promutagenic (Ames test).

- hypoallergenic (RIPT test).

cf. Annex pp.42-45.

EFFECTIVENESS EVALUATION

LIGHTOCEANE®

Marine skin-lightener interfering with different steps in melanogenesis

Before melanin synthesis

Reduction of melanogenic mediators production	Reduction of nitric oxide (NO) release *In vitro* test on human keratinocytes

During melanin synthesis

Inhibition of melanogenesis	Inhibition of mushroom tyrosinase activity *In tubo* assay
	Inhibition of the melanin synthesis *In vitro* study on reconstructed pigmented skins
	Inhibition of tyrosinase activity *Ex vivo* histological study on skin human explants

After melanin synthesis

Control of melanosome transfer to keratinocytes	Inhibition of the dendricity of melanocytes Inhibition of TRP-1 expression *Ex vivo* histological study on skin human explants

With preventive protection

Protection against free radicals	Scavenger effect DPPH assay
Protection against UVA & UVB	Preventive cytoprotection *In vitro* tests on keratinocytes & reconstructed skins
Inhibition of inflammation	Inhibition of 5-lipoxygenase *In tubo* assay
	Inhibition of IL 1 alpha *In vitro* test on reconstructed skins

& additional properties

Improvement of cell metabolism	Increase of cell proliferation *Ex vivo* study on human keratinocytes
Protection of the dermal matrix fibres against proteases deterioration	Anti-collagenase activity *In tubo* assay
	Anti-elastase activity *In tubo* assay

Potent skin lightener from the Ocean

LIGHTOCEANE® influences all main pathways involved in melanogenesis

Action before melanin synthesis

Exposure to UV light induces the keratinocytes to produce several mediators which affect melanocytes. As a consequence, melanocytes proliferate and show greater dendricity.

So, undue pigmentation could be suppressed by inhibiting or neutralizing mediator action.

Reduction of the production of the melanogenic mediator: NO

It is well proved that in response to UVA and UVB radiation, normal human keratinocytes secrete nitric oxide (NO).

In vitro NO released from UV-exposed keratinocytes increases melanogenesis in human melanocytes suggesting that NO is a potent keratinocyte-derived mediator of melanogenesis (Romero-Graillet C. *et al*., 1996 – J. Biol. Chem. 271 : 28052-28056 ; 1997 – J. Clin. Invest. 99: 635-642).

On the basis of these scientific data, the purpose of this study is to determine is LIGHTOCEANE® is able to reduce the release of NO in UV-induced keratinocytes, and consequently to down regulate UV melanogenesis.

Method

Human keratinocytes are irradiated with UVB at 15 mJ.cm^2 and 30 mJ.cm^2 that allows a release of NO.

The total nitric oxide (NO) present in the culture medium after 72 h cultivation has been detected by using the assay kit (Assay designs/Stressgen).

This kit involves the enzymatic conversion of nitrate to nitrite, by the enzyme nitrate reductase, followed by the colorimetric detection of nitrite as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm.

It allows for the total determination of both NO products by conversion of the sample nitrate into nitrite, followed by the determination of the total concentration of nitrite in the culture medium.

The concentrations of total nitric oxide in samples are evaluated from the graph obtained from each standard with

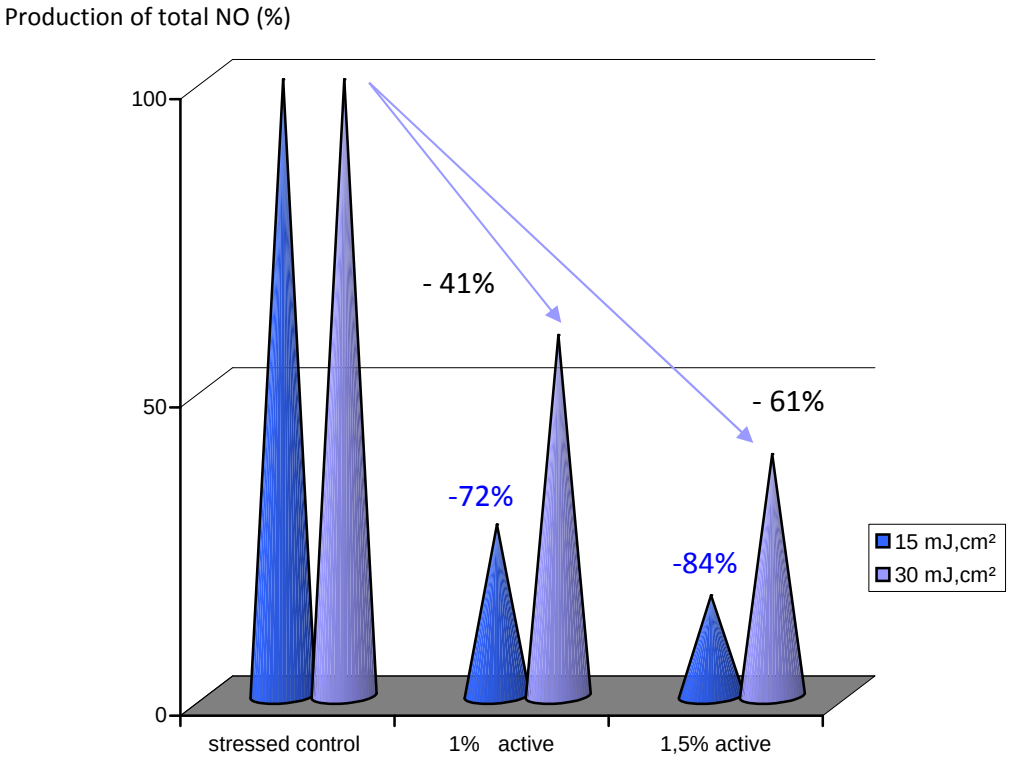
$$y = 0.001\,937\,x + 0.0809 \qquad R^2 = 0.8279$$

In each experiment, LIGHTOCEANE® is tested at 1% and 1.5%.

Results

In both experiments results express lower amounts of total NO production in comparison with stressed control (maximum expression = 100 %).

At 1%, LIGHTOCEANE® is capable to reduce by 72 % the release of nitric oxide in response to a dose of 15 mJ.cm² UVB.



➤ LIGHTOCEANE® is able to reduce the release of NO that stimulates tyrosinase

Therefore the activation of UV melanogenesis is inhibited before it starts.

Action during melanin synthesis

Tyrosinase plays a central role in skin pigmentation by catalyzing the first two stages in the melanin synthesis *i.e.* the conversion of the tyrosinase :

- into DOPA (dihydroxy-phenylalanine) and then
- into DOPAchrome.

The DOPAchrome is then oxidized under the effect of other enzymes and polymerized into melanin.

Melanin determines the color of the skin.

So, products capable of inhibiting tyrosinase activity are developed to inhibit skin pigmentation.

Inhibition of melanogenesis

The ability of LIGHTOCEANE® to inhibit melanogenesis is evaluated by several methods:

- the tyrosinase inhibition by i*n tubo* test
- the inhibition of melanin synthesis by *in vitro* test on reconstructed pigmented skins (Skin Ethic model)
- the inhibition of tyrosinase activity by *ex vivo* studies on skin human explants.

Inhibition of the mushroom tyrosinase activity

In tubo test

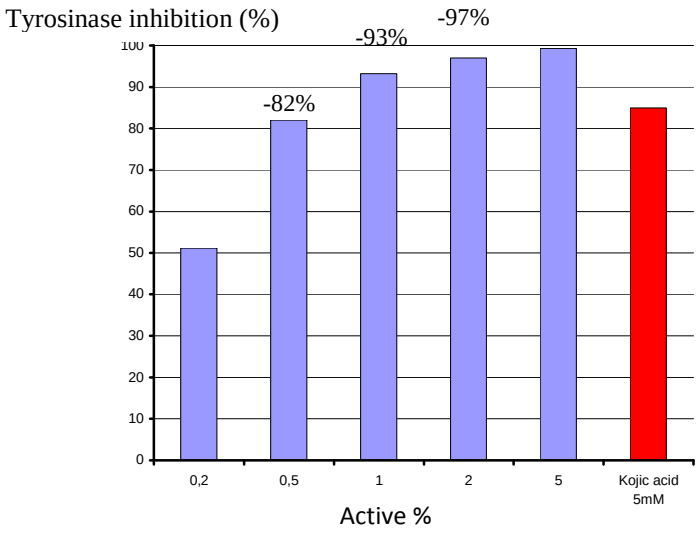
Method

The activity of tyrosinase is quantified by monitoring the formation of DOPAchrome by measuring the optical density of LIGHTOCEANE® at a wavelength of 475 nm. The substrate of the enzyme is L-tyrosine and the reaction requires the presence of the cosubstrate L-DOPA.

Several concentrations of LIGHTOCEANE® are tested (0.2– 0.5 – 1 – 2 and 5 %). Kojic acid is dosed at 5mM.

Results

Results represent the average of three experiments for each active tested ($\alpha = 0.05$).
They are illustrated below.



The addition of LIGHTOCEANE® leads to a dose dependent inhibition of DOPAchrome fraction.

The IC50 (extract concen-tration which produces 50% inhibition of the enzyme activity) is about 0.2 % active.

At 1 % LIGHTOCEANE® inhibits to more than 90 % tyrosinase activity.

➤ LIGHTOCEANE® is a potent inhibitor of tyrosinase with a dose dependant action.

Inhibition of the melanin synthesis

It is well documented that the melanin synthesis is related to:

-an increase of tyrosinase activity (Friedmann P.S. & Glichrest , B.A., 1987 – J.Cell Physiol. 133 : 88-94 ; Ramirez-Bosca A. *et al.,* 1992 – Arch. Dermatol. Res. 284: 358-362) and to

-an increase of tyrosinase expression (Gilchrest B.A. *et al.,* 1996 – Photochem. Photobiol. 93 : 1-10).

In vitro study on reconstructed pigmented skins

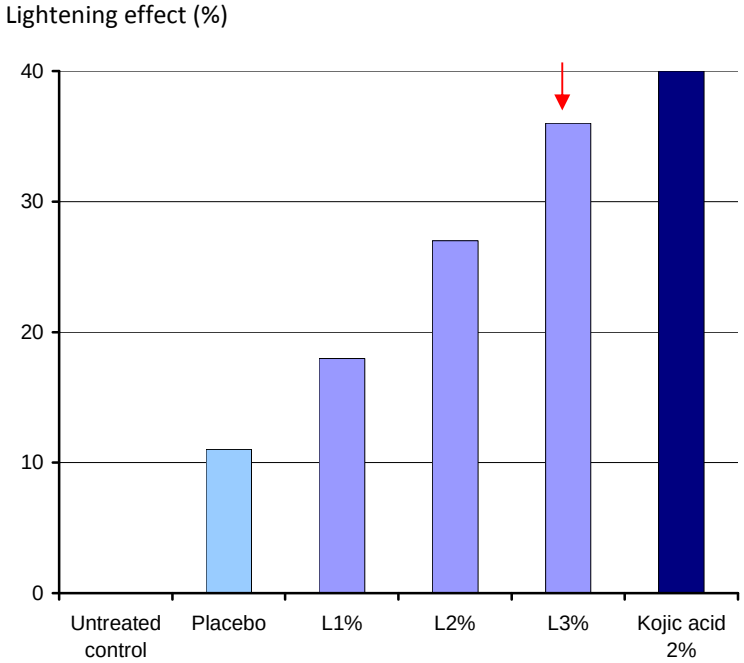
Method

The lightening activity of LIGHTOCEANE® formulated at 1 – 2 or 3 % has also been confirmed on reconstructed pigmented skins (RHPE –Skin Ethic model) after 4 days of topical application. Its activity is compared with that of kojic acid at 2 % in the same kind of formulation.

The evaluation of melanin content has been performed at day 7.

Results

LIGHTOCEANE® (L 3 %) shows a "measurable "lightening effect at the surface of the skin at the end of experiment.



At 3 % LIGHTOCEANE® induces a depigmenting activity of 36 %

compared to untreated control.

➤ LIGHTOCEANE® is a potent inhibitor of melanin synthesis with a dose dependant action.

Inhibition of the tyrosinase activity

The pathway of melanin synthesis is catalyzed by different enzymes.

Tyrosinase is the key enzyme responsible for UVB-induced melanogenesis. It plays a critical role in multiple reactions steps whereas DOPAchrome tautomerase (TRP-2) and DHICA oxidase (TRP-1) are vital in the conversion from DOPAchrome to 5,6-dihydroxyindole-2-carboxy acid , which leads to the final formation of the eumelanin.

So, products capable of inhibiting such enzymes activities can lead to reduction in melanin formation and therefore skin lightening.

Ex vivo study on human skin explants

This study has been performed on skin human explants by the French testing company BIO-EC Laboratory (91165- Longjumeau).

Method

LIGHTOCEANE® is formulated at 2 % in an excipient known to bring none lightening effect.

Human skin explants are kept alive in a specific medium: BEM (BIO-EC's Explants Medium). They are treated following various conditions in different batches:

Control (Plastie)	**T0**	3 explants
Control	**T**	6 explants
Formulation assay	**F**	6 explants
Control + UV	**TUV**	6 explants
Formulation assay + UV	**FUV**	6 explants.

1. *Treatment*

At Day O (J0), skin explants are kept alive in 2ml culture medium. The formulation is applied topically each two days . Controls received no treatment.

2. *Chronic UVA/UVB irradiations*

Samples « T » and « F » received none irradiation.

Samples « TUV » and « FUV » received each day a UVA dose of 1,125 J/cm² (+ 6-8% d'UVB), corresponding to the solar irradiation spectrum.

Irradiations are performed 30 minutes before treatment with active (in order to avoid an eventual filter effect) at differents days: J1, J2, J3, J4, J5, J6, J7, J8 and J9.

After irradiations in the presence of active, skin explants are kept alive again.

3. *Sample removal*

At J6, 3 skin explants of each batch are taken off and cut into two pieces. One piece is fixed in buffered formaldehyde and the second frozen at -80°C.

At J10, the 3 remaining explants of each batch are taken off and cut into two pieces and prepared as previously.

4. Histological studies

After 24h fixation in formaldehyde, some samples are prepared for observations by optical microscopy (microscope Leica type DMLB, objectif x 40). Photographies are performed by using a camera tri CCD Sony DXC 390P.

The general morphology has been observed using the Masson trichome staining technique.

Visualization of the presence of melanin in the epidermis of the skin samples is carried out using the Fontana-Masson staining technique which allows follow melanogenesis during the study.

Results

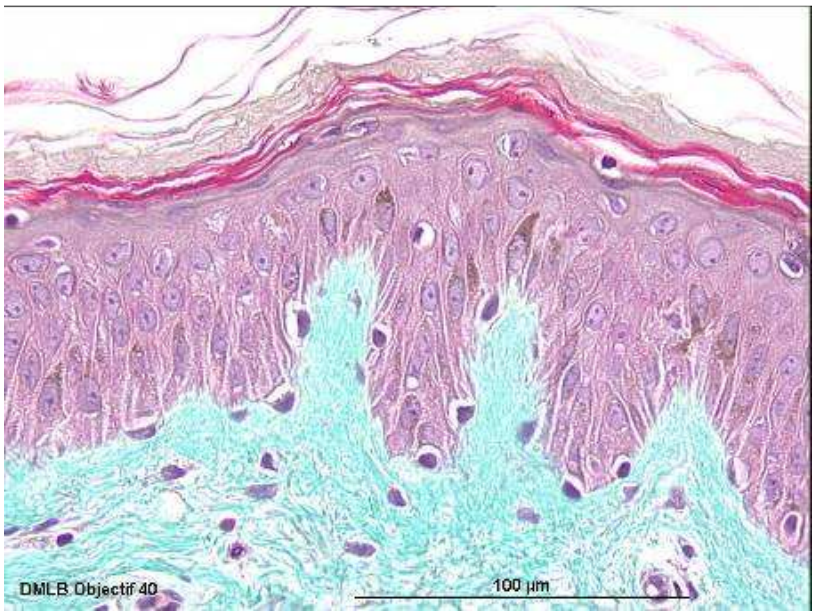
Results relative to the morphological study are illustrated on Plate 1 to 5. They are distributed as follows :

Pl. 1 control at day 0 (**T J0**)
Pl. 2 control no treatment and irradiation at day 6 (**TUV J6**)
Pl. 3 treatment 2 % LIGHTOCEANE® and irradiation at day 6 (**FUV J6**)
Pl 4 control no treatment and irradiation at day 10 (**TUV J10**)
Pl. 5 treatment 2 % LIGHTOCEANE® and irradiation at day 10 (**FUV J10**).

Pl. 1 - control at day 0 (T J0)

The *stratum cornuem* is less laminated.
The epidermis is constituted of 4-5 cell layers showing with a good morphology.
The dermo-epidermal junction is clearly apparent.
The dermis contains thick collagen fibers constituting a quite enough dense network. Cells are clearly visible.

Pl. 2- control no treatment and irradiation at day 6 (TUV J6)

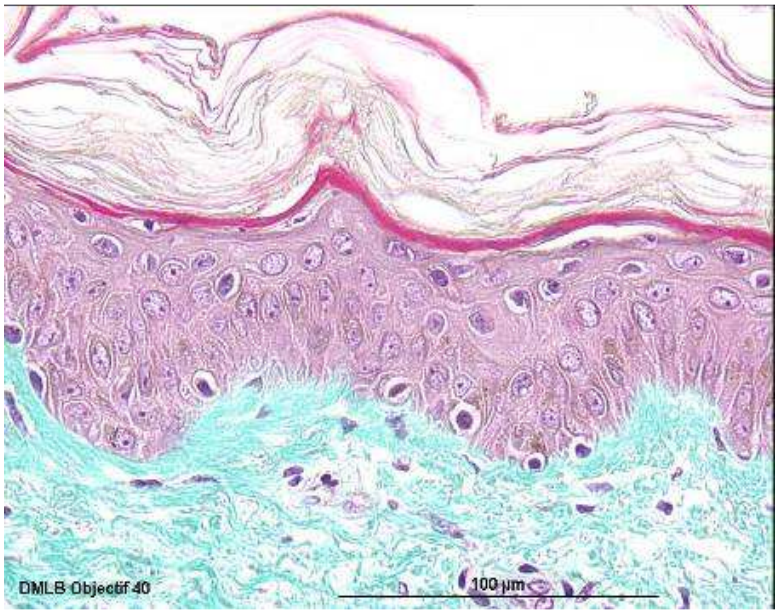


The *stratum corneum* appears slightly thick and keratinised in surface. Parakeratose is reduced.
The epidermis contains 4-5 cell layers with well preserved morphology.
The dermo epidermal junction is apparent. The dermis contains cells and numerous thick collagen fibers constituting a dense network. Cells are numerous.

Pl. 3- treatment with 2 % active and irradiation at day 6 (FVU J6)

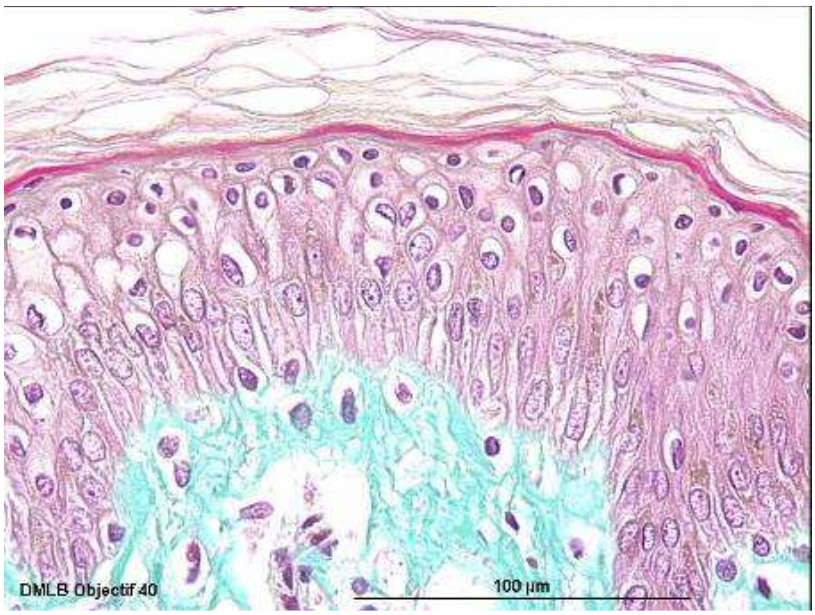
The *stratum corneum* appears thick, keratinized in surface. Parakeratose is reduced.
The epidermis contains 5-6 cell layers with well preserved morphology. Spongiose is reduced in basal layers.
The dermo epidermal junction is apparent. The dermis contains cells and numerous thick collagen fibers constituting a dense network.

Pl. 4 - control no treatment and irradiation at day 10 (TUV J10)



The epidermis is constituted of 4-5 cell layers. Some cells with pycnotic nucleus are visible in the upper epidermal cell layers. Spongiose is moderated in the basal part. The dermo-epidermal junction is apparent.
The dermis contains thick collagen fibers constituting a variously dense network. Cells are clearly visible.

Pl. 5 - treatment with 2 % active and irradiation at day 10 (FUV J10)



The *stratum corneum* is thick, well laminated.
The epidermis contains 4-5 cell layers. Some cells appears with oedema and pycnotic nucleus, in the *spinosum stratum*. The relief of the dermo-epidermal junction is moderated.
The dermis contains thick collagen fibers constituting a variously dense network. Cells are clearly visible.

➤ LIGHTOCEANE® does not induce important morphological changes of the cell structure of skin explants at day 6 as at day 10.

Results relative to the melanin synthesis are illustrated on next pages. They are distributed as follow :

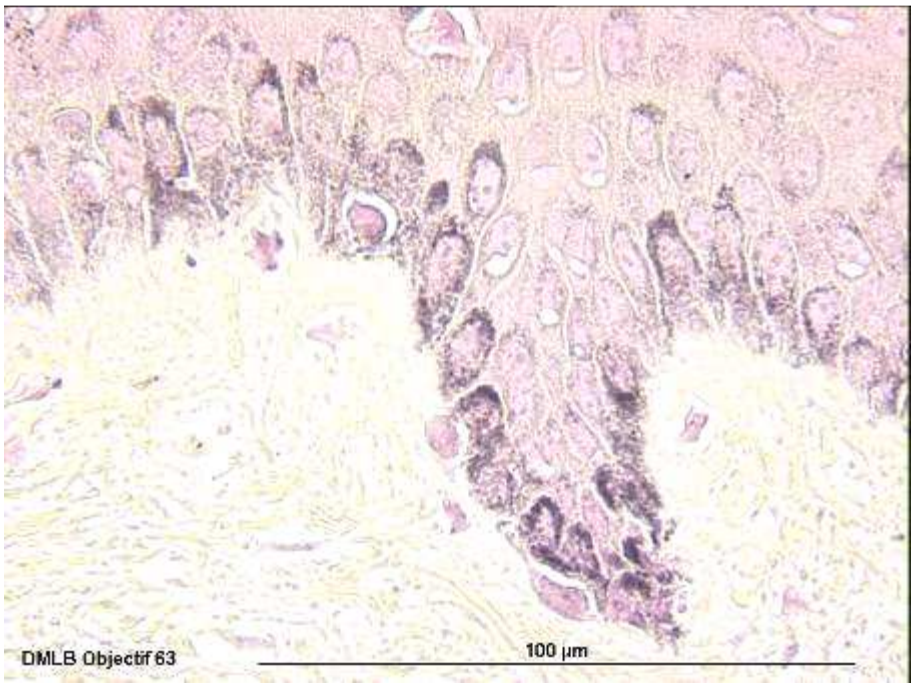
Pl. 6	control at day 0 (**T J0**)
Pl. 7	control no treatment and irradiation at day 6 (**TUV J6**)
Pl. 8	treatment 2 % LIGHTOCEANE® and irradiation at day 6 (**FUV J6**)
Pl. 9	control no treatment and irradiation at day 10 (**TUV J10**)
Pl.10	treatment 2 % LIGHTOCEANE® and irradiation at day 10 (**FUV J10**).

These results enable us to conclude that melanin synthesis induced by UV irradiation (30 minutes) each 9 days is attenuated when LIGHTOCEANE® is applied each 2 days after irradiation.

This effect is even noticeable as early as day 6 irradiation and more important at day 10.

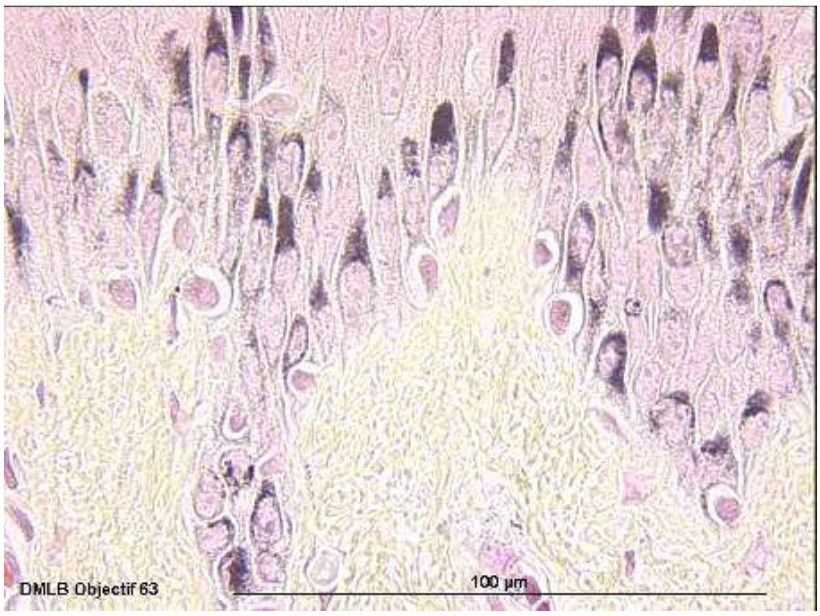
➤ LIGHTOCEANE® inhibits melanin synthesis & thus decreases skin pigmentation.

Pl. 6 - control at day 0 (T J0)



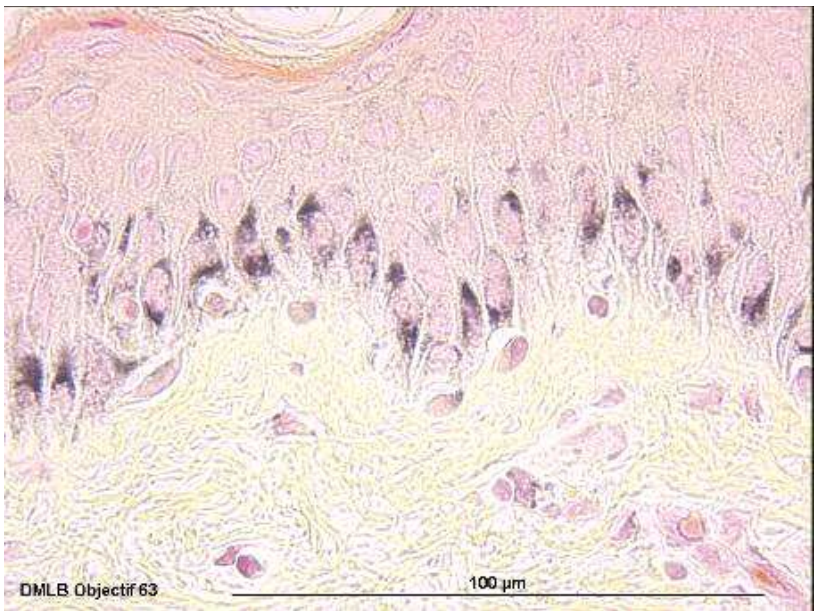
The morphology of melanocytes is good.
Melanin content and dendricity are moderated as the contact with keratinocytes.
Melanin transfer is restrained.
The amount of melanin in the basal layer is clearly apparent whereas those of supra-basal layers are reduced.

Pl. 7 - control no treatment and irradiation at day 6 (TUV J6)



This batch shows (1) light increase of melanin content in melanocytes and (2) marked increase of dendricity and of keratinocytes contact and of melanin transfer.
A pro-pigmentation activity is effective.

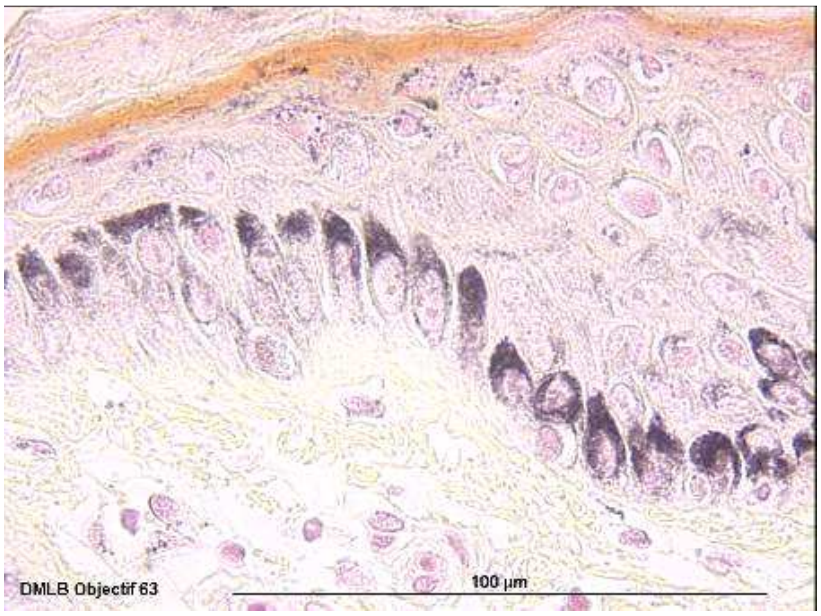
Pl. 8 - treatment with 2 % active and irradiation at day 6 (FUV J6)



Compared to TUV J6, it appears marked decrease of dendricity, of keratinocytes contact and melanin transfer.

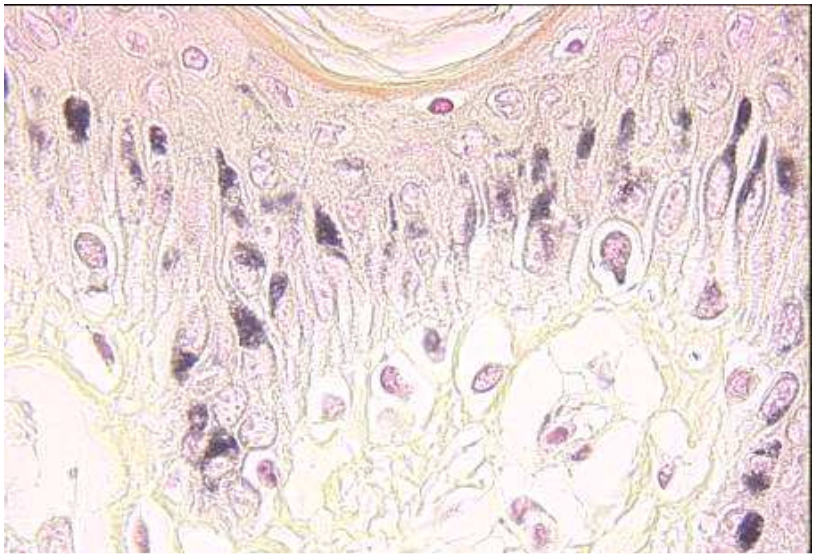
➤ that proves the depigmenting activity of LIGHTOCEANE®
as early as 6 days of experiment.

Pl. 9 - control no treatment and irradiation at day 10 (TUV J10)



It appears marked increases of melanin content in melanocytes, of contact with keratinocytes and of melanin transfer.
The dendricity and the melanin content in the basal and supra-basal parts increase slightly.
Irradiation induces a pro-pigmentation activity.

Pl. 10 - treatment with 2 % active and irradiation at day 10 (FUV J10)



Compared to TUV J10, it appears (1) a moderated decrease of melanin content in melanocytes and of dendricity and (2) a marked decrease on keratinocytes contact and of melanin transfer.

➤ LIGHTOCEANE® induces clearly a depigmentation action after 10 days treatment.

Action after melanin synthesis

Melanosomes synthesized within melanocytes are transferred to keratinocytes through melanocyte dendrites. These dendrites transfer melanin to keratinocytes in response to hormone (*e.g.* melanocyte stimulating hormone: MSH) and UV light, both of which stimulate melanin synthesis and melanosome transfer (Virador V.M. *& al*., 2002 – FASEB J. 16 : 105-107).

During this process, dendrite extension is necessary. This results in a constant supply of melanin reaching the epidermis. So, in this way, skin pigmentation is determined.

Melanosomes express different proteins such as TRP-1, TRP-2 and P-protein which are essential for melanin synthesis.

Control of mature melanosome transfer to keratinocytes

We have chosen to study the action of LIGHTOCEANE® on the expression of TRP-1 (DHICA-oxidase)

TRP-1 (tyrosinase-related protein-1) forms in the melanosomal membrane a multimeric complex which is thought to play a part in :

- the protective role of the tyrosinase against oxidants and /or in the glycosylation,

- the tyrosinase proliferation, morphology and activity of the melanocytes and melanosomal cells (Hara, H. *& al.*, 1994 – J. Invest. Dermatol., 102:495-500); Li Cy *& al.*, 2004- Br. J. Dermatol., 150 (6) : 1081-1090).

TRP-1 promotes further oxidation and polymerization of DHICA melanins (brown melanins) that are eumelanins known to provide a better photoprotection than yellowish-red phaemelanins (Petit, L. & Pierard, G.E., 2003-Int.J. Cosmetic. Sc. 25 : 169-181).

Inhibition of the TRP-1 activity

Ex vivo study on human skin explants

This immuno labelling study on the expression of TRP-1 has been performed by the French testing company BIO-EC Laboratory (91165- Longjumeau- France).

Method

Skin explants are irradiated and prepared as previously (pp.16-17). They are studied at day 0, day 6 and day 10 .

LIGHTOCEANE® is applied at 2 % in the same formulation as previously.

The « Tyrosinase related Protein 1 » have been labelled on frozen sections with an anti-TRP1 monoclonal, clone Ta99 (Covance ref. SIG-3815 –1000) .

Nucleus are colored with Masson staining technique.

Results

Results are illustrated on next pages as follows :

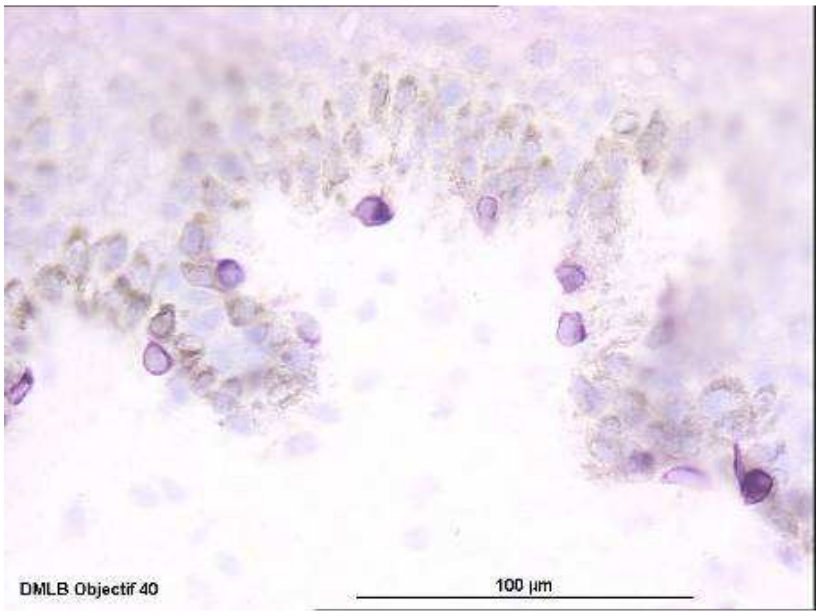
Pl. 11 control at day 0 (T J0)
Pl. 12 control no treatment and no irradiation at day 6 (T J6)
Pl. 13 control no treatment and no irradiation at day 10 (T J10)
Pl. 14 control no treatment and irradiation at day 6 (TUV J6)
Pl. 15 treatment 2 % LIGHTOCEANE® and irradiation at day 6 (FUV J6)
Pl. 16 control no treatment and irradiation at day 10 (TUV J10)
Pl. 17 treatment 2 % LIGHTOCEANE® and irradiation at day 10 (FUV J10).

At day 6 as at day 10, the chronic UV irradiation increases the dendricity of positive TRP-1 melanocytes.

➤ The treatment with 2 % LIGHTOCEANE® under chronic UV irradiation inhibits practically completely the dendricity of melanocytes at 10 days of experiment.

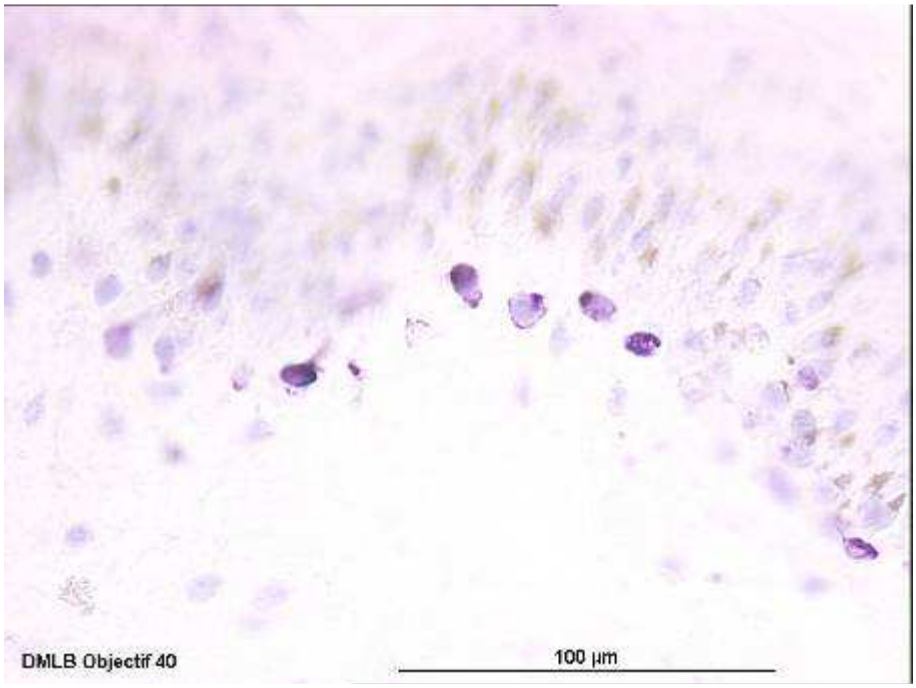
➤ This reduction of dendricity of melanocytes is reduced as early as 6 days of experiment.

Pl. 11 - control at day 0 (T0)



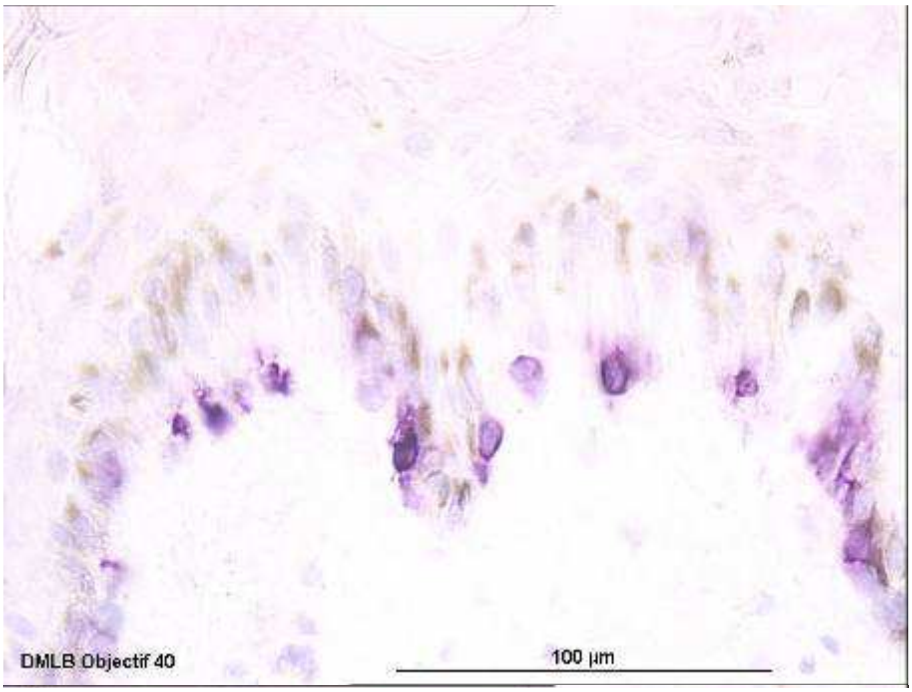
The dendricity of positive TRP-1 melanocytes appears reduced to very limited.

Pl. 12 - control no treatment and no irradiation at day 6 (T J6)



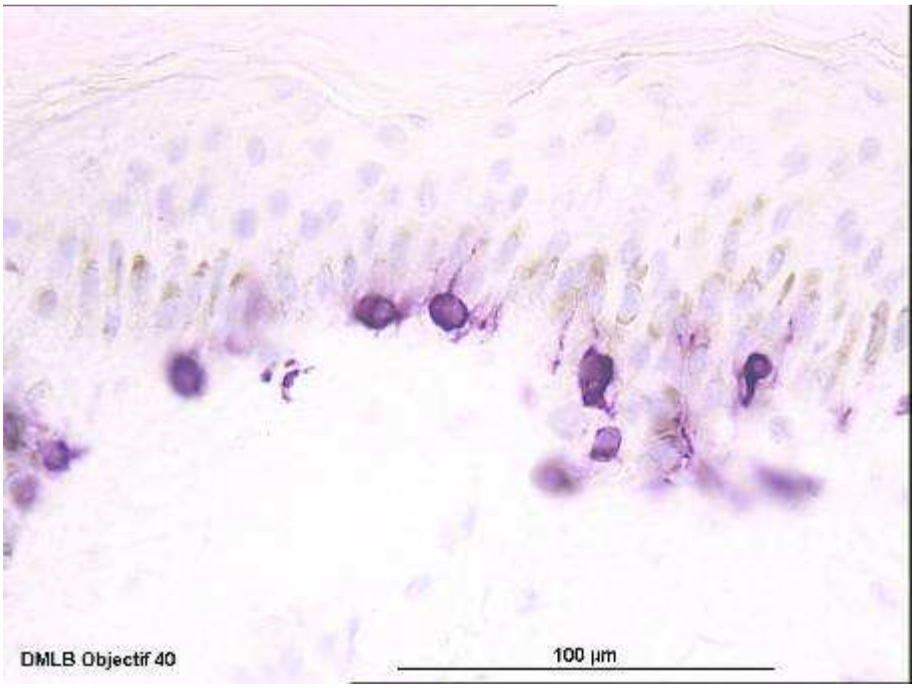
The dendricity of TRP-1 positive melanocytes appears slight to very limited.

Pl. 13 - control no treatment and no irradiation at day 10 (TJ10)



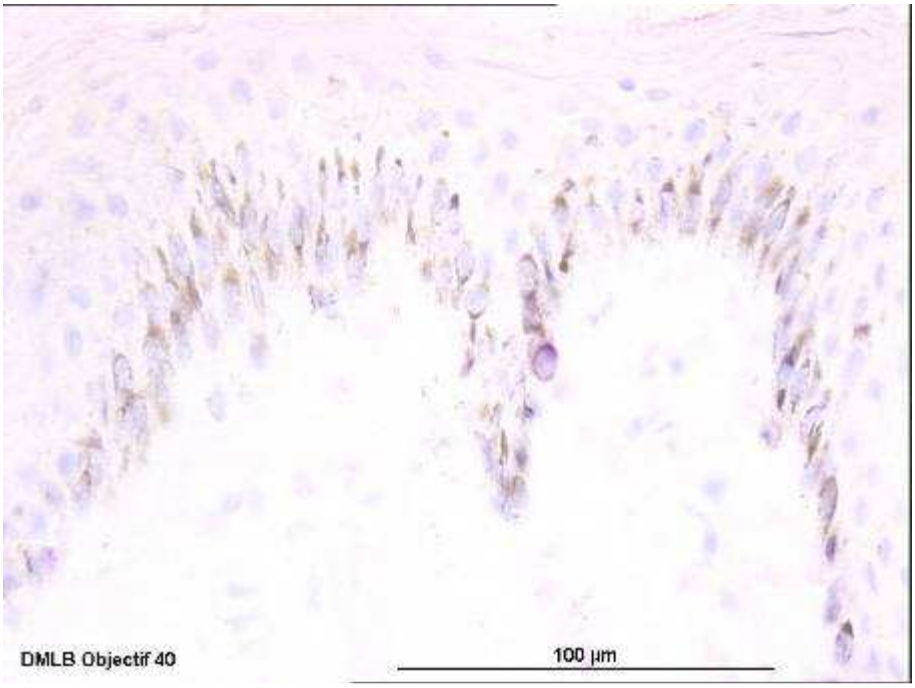
The dendricity of positive TRP-1 melanocytes is reduced.

Pl. 14 - control no treatment and irradiation at day 6 (TUVJ6)



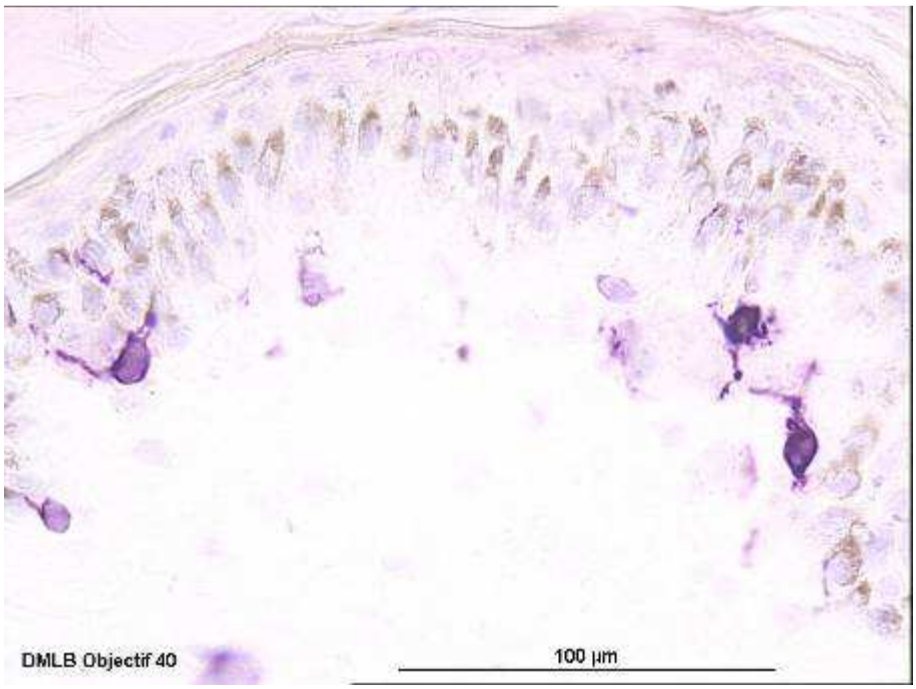
The dendricty of positive TRP-1 melanocytes is apparent.

Pl. 15 - treatment with 2 % active and irradiation at day 6 (FUV J6)



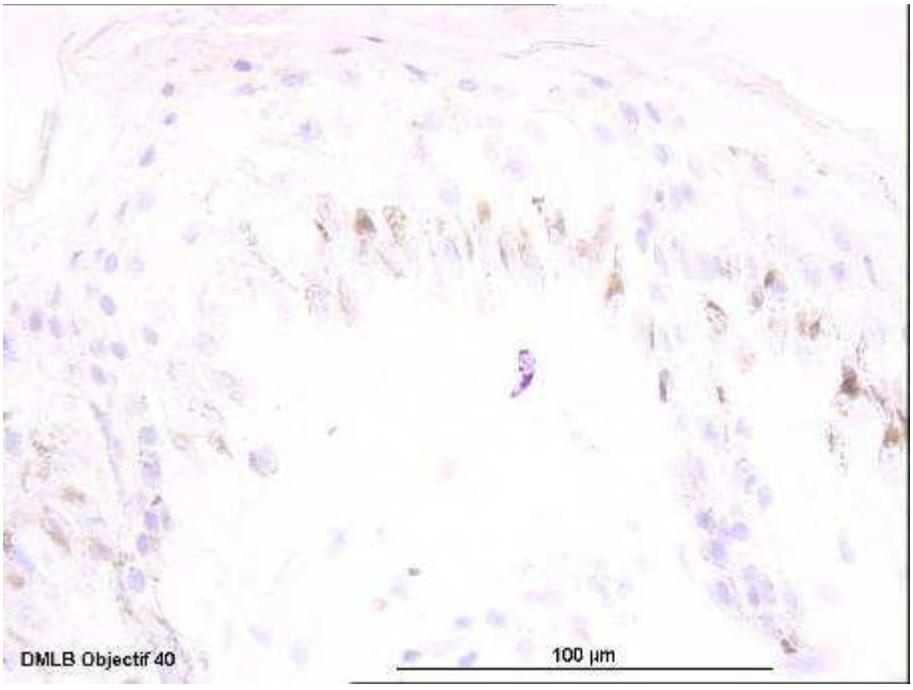
The dendricity of the positive TRP-1 melanocytes is reduced..

Pl. 16 - control no treatment and irradiation at day 10 (TUVJ10)



The dendricty of positive TRP1 melanocytes is clearly apparent.

Pl. 17 - treatment with 2 % active and irradiation at day 10 (FUVJ10)



The dendricity of the positive TRP-1 melanocytes is very minimized.

Evaluation of the number of the positive TRP-1 cells

Methods

The number of anti-TRP-1 labelled cells is evaluated for each sample by using the application software Leica Qwin. (evaluation by cm of epidermis). LIGHTOCEANE® is applied at 2 % in the same formulation that previously (F – *cf* p.16).

Results

Results are shown on the tables below and on the graph on next page.

Number of the positive TRP-1 cells / cm epidermis

	T0	TJ6	TUVJ6	FUV J6	TJ10	TUVJ10	FUV J10
	8.3	29.1	63.7	51.8	27.4	58.8	1.0
	16.4	24.1	47.1	52.7	38.5	74.2	1.9
	12.2	35.6	59.8	29.1	36.0	55.3	1.0
Mean	**12.32**	**29.59**	**56.89**	**44.54**	**33.93**	**62.76**	**1.31**
Standard deviation	3.29	4.69	7.08	10.91	4.75	8.19	0.42
% variation / T		**140**	**92**	**-22**	**175**	**85**	**-98**

Statistical analysis : **Student test**

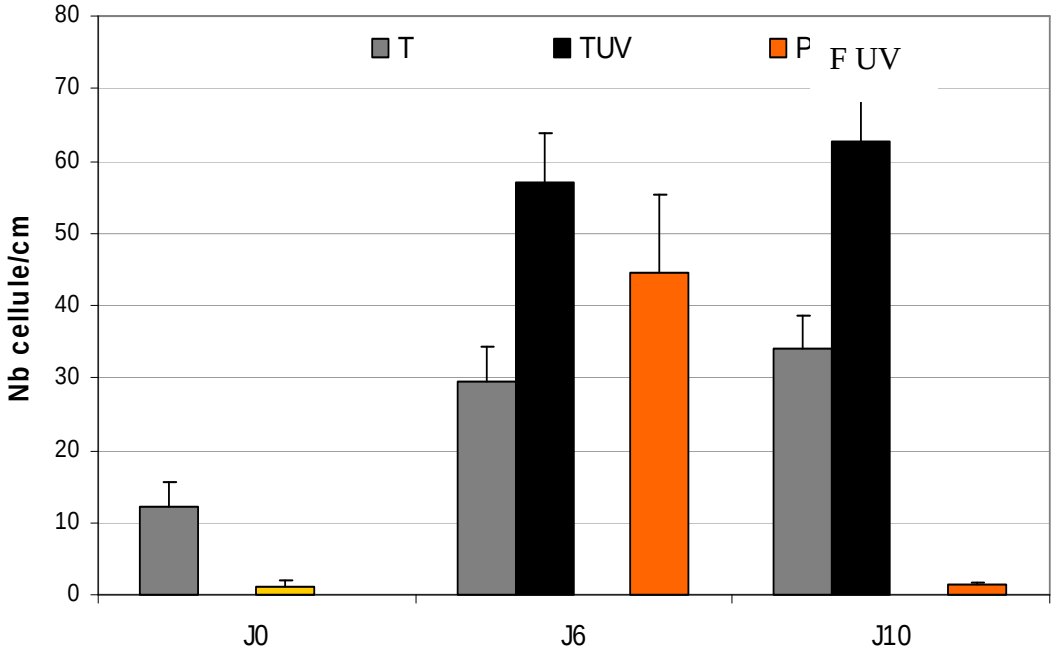
	T0	TJ6	TUVJ6	FUVJ6	TJ10	TUVJ10	FUVJ10
T0	1.000	**0.016**	**0.005**	**0.043**	**0.008**	**0.006**	**0.040**
TJ6	**0.016**	1.000	**0.014**	0.183	0.410	**0.014**	**0.013**
TUVJ6	**0.005**	**0.014**	1.000	0.262	**0.024**	0.487	**0.008**
FUVJ6	**0.043**	0.183	0.262	1.000	0.304	0.138	**0.030**
TJ10	**0.008**	0.410	**0.024**	0.304	1.000	**0.020**	**0.010**
TUVJ10	**0.006**	**0.014**	0.487	0.138	**0.020**	1.000	**0.009**
FUVJ10	**0.040**	**0.013**	**0.008**	**0.030**	**0.010**	**0.009**	1.000

Significant effect when $p < 0.05$.

In summary

Cell/cm epidermis	**Day 0 (J0)**		**Day 6 (J6)**		**Day 10 (J10)**	
	Mean	Standard deviation	Mean	Standard deviation	Mean	Standard deviation
T	**12.3**	3.3	**29.6**	4.7	**33.9**	4.8
TUV			**56.9**	7.1	**62.8**	8.2
FUV			**44.5**	10.9	**1.3**	0.4

Number of the positive TRP-1 cells / cm epidermis



At day 6 (J6)

The chronic exposure to UV during 6 days (TUV J6) induces a significant increase of 92 % of the number of positive TRP-1 cells, in comparison with the no irradiated control at day 6. (T J6).

The UV exposure and the topic treatment with 2 % LIGHTOCEANE (FUV J6) during 6 days induce a non significant decrease of 22 % of the number of positive TRP-1 cells, in comparison to the irradiated control (TUV J6).

At day 10 (J10)

The chronic exposure to UV during 10 days (TUV J10) induces a significant increase of 85 % of the number of positive TRP-1 cells, in comparison to the no irradiated control at day 10.(T J10).

The UV exposure (30 min/day for 9 days) and the topic treatment with 2 % LIGHTOCEANE (applied each two days) during 10 days (FUV J10) induce at day10 a significant decrease of 98 % of the number of positive TRP-1 cells , in comparison to the irradiated control (TUV J10).

➤ At 2 % LIGHTOCEANE® exhibits a significant inhibitory effect on DHICA-oxidase (TRP-1) which is implied in the synthesis of eumelanins (brown melanins).

➤ At 2 % LIGHTOCEANE® is able to inhibit melanosome transfer to keratinocytes by action on melanocyte dendricity and therefore reduces skin pigmentation.

LIGHTOCEANE® protects against free radicals and UVA-UVB irradiations

LIGHTOCEANE® is able to minimize the action of certain triggers before the pigmentation is carried out. It brings a preventive action by:

- combating free radicals
- providing an effective protection against UV radiation
- fighting inflammation process.

Protection against free radicals

Free radicals contribute to tyrosinase activation and melanin formation.

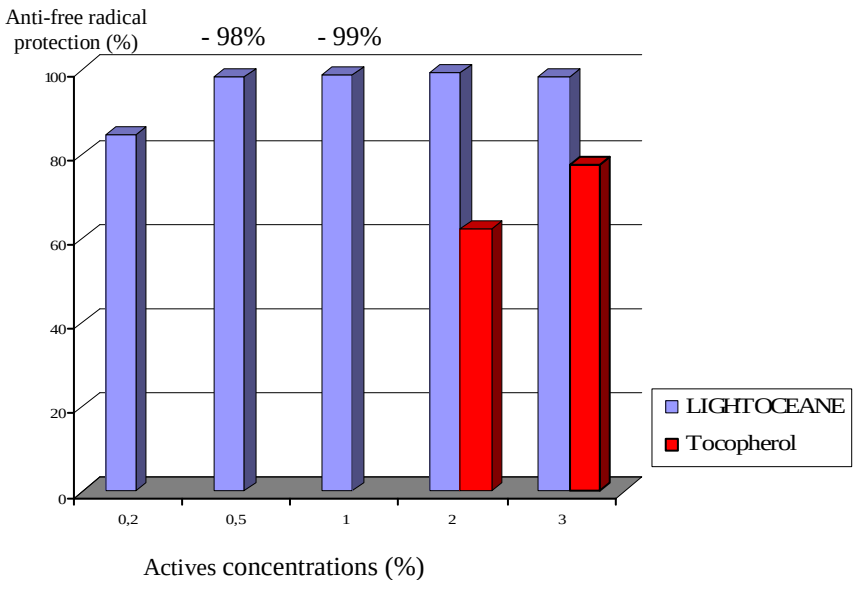
Methods

The anti-free radical properties of LIGHTOCEANE® are evaluated by *in tubo* chemical test: DPPH test. DPPH (diphenyl picryl hydrazyl) is a stable, free violet-colored radical which is modified in its leuco compound by the substances that trap the free radicals (scavenger effect).

A solution of the purple coloured DPPH-radical is mixed with the test active. The decrease of the absorption is determined photometrically. The standard is tocopherol (concentration solution : 10^{-3} M).

Results

Results are given in percent inhibition in relation to control without any active ingredient (average of 2 assays).



➤ LIGHTOCEANE® is a powerful scavenger of free radicals.

Its anti-free radical performance reaches 99 % with 1 %.

Protection against UV radiations

UV radiations are the major trigger of melanogenesis. They act directly on melanocytes to boost melanin production. They also induce the generation of reactive oxygen species in the cells.

The DPPH assay is completed by *in vitro* tests on human keratinocytes in order to evaluate an eventual cytoprotective effect of LIGHTOCEANE® on the cells submitted to UVA and UVB.

Protection against UVB

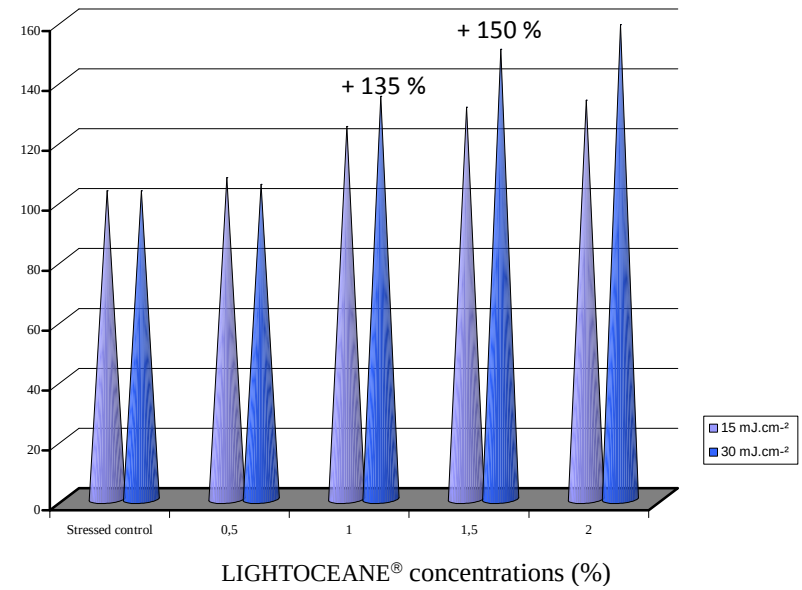
In vitro study on human keratinocytes

Methods

Human keratinocytes were seeded at 8000 cells per well (96-well microtiter plate) and grown in an optimal medium for 24 h before addition of the active.
LIGHTOCEANE® is tested at 4 concentrations : 0.5 - 1 - 1.5 & 2 %).
After 48 h incubation, cells were washed with the saline buffer PBS and submitted to UVB irradiation (2 doses : 15 mJ/cm² and 30 mJ/cm²).
After irradiation, cells were washed in PBS again. Media were added (with or without active). After 48 h cultivation, the viability was determined by the MTT test.

Results

Results are expressed as percentage against control (without ingredient). They represent the average of 2 experiments with 4 values for control and each concentration.



Results are validated statistically by
two methods :

- the analysis of variance ANOVA (fix model) with υ1=4 and υ2=20

  Experiment 15 mj.cm-² :
  α : 0.001
  Fα : 14.819
  Fc : 40.871 ***

  Experiment 30 mj.cm-² :
  α : 0.001
  Fα : 14.819
  Fc : 51.867 ***

- the least significant difference with
  Δc= | m1-m2| and
  Δ =tαV [(2. υi)/2]
  υI : intrinsic variance.

Control=0.5 %< <1 %=1.5 %=2 %

The protective effect of LIGHTOCEANE® against UVB appears significant to highly significant as early as 1 % active whatever the UVB dose.

In vitro study on reconstructed skins

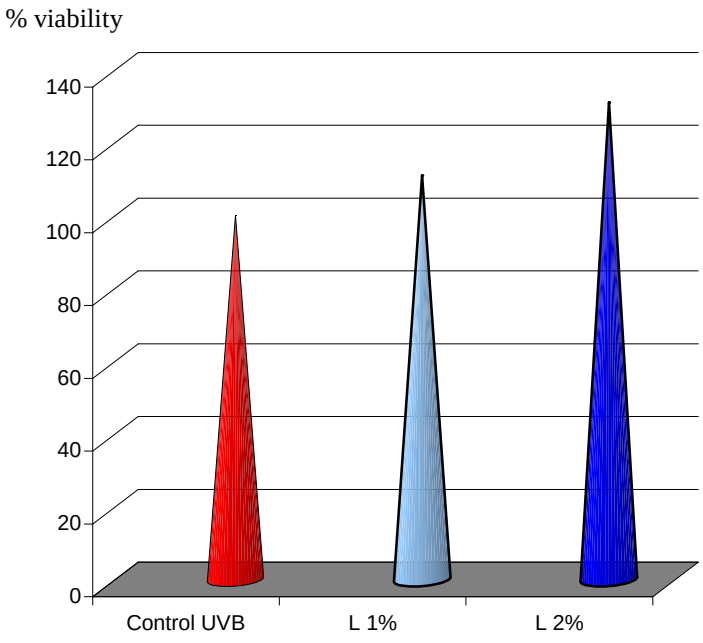
The protective potential of LIGHTOCEANE® has been confirmed on reconstructed skins

Methods

Reconstituted skins (Skin Ethic model) are cultured for 5 hours in the presence (1 % or 2 %) or the absence of LIGHTOCEANE®.
The UVB irradiation (dose: 300 mJ/cm²) is performed on skins in PBS only.
The MTT test performed 72 h after irradiation. Optical density is measured in triplicate.

Results

This graph shows that 2 % LIGHTOCEANE® increases by 31 % the viability of reconstructed skins, compared to irradiated control (in red).



Results are validated statistically by two methods :

➤ the analysis of variance ANOVA (fix model) with υ1=2 and υ2=18

α : 0.001
Fα : 10.39
Fc : 22.527 ***

➤ the least significant difference:

TUV	L1	L2
0.096	< 0.107	<< 0.126

L1 % : significant compared to TUV

L2 % : highly significant compared to L1 %.

➤ At 2 % LIGHTOCEANE® induces a very highly significant protection against UVB.

Protection against UVA

In vitro study on human keratinocytes

Methods

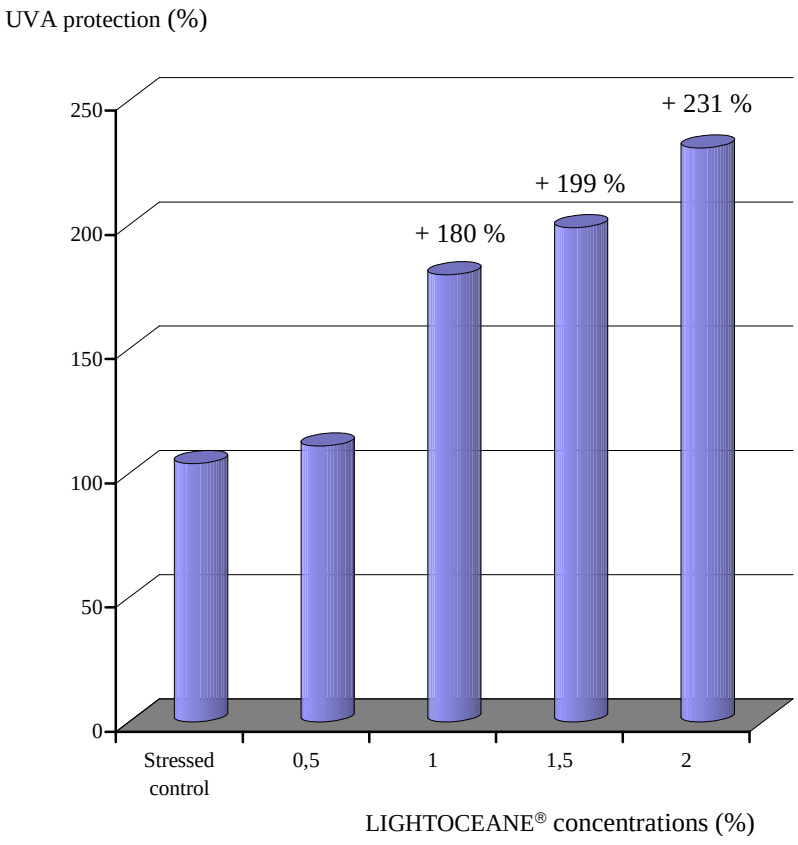
Human keratinocytes were seeded at 8000 cells per well (96-well microtiter plate) and grown in an optimal medium for 24 h before addition of the active.

LIGHTOCEANE® is tested at 4 concentrations : 0.5 - 1 - 1.5 & 2 %).

After 48 h incubation, cells were washed with the saline buffer PBS and submitted to UVA irradiation (1 dose : 15 J/cm²).
After irradiation, cells were washed in PBS again. Media were added (with or without ingredient). After 48 h cultivation, the viability was determined by the MTT assay.

Results

Results are expressed as percentage against control (without ingredient). They represent the average of 2 experiments with 4 values for control and each concentration.



Results are validated statistically by two methods :

➤ the analysis of variance ANOVA (fix model) with υ1=4 and υ2=20

α : 0.001
Fα : 14.819
Fc : 117 ***

➤ the least significant difference with
Δc= | m1-m2| and
Δ =tαV[(2. υi)/2]
υI : intrinsic variance.

C =0.5 % <<< 1 % = 1.5 % = 2 %
C: control.

➤ The protective effect of LIGHTOCEANE® against UVA is significant to highly significant as early as 1 % active.

LIGHTOCEANE® fights inflammation

Two major causes of inflammation exist: irritants and UV irradiation, both generates reactive oxygen species in the cells. Moreover UV irradiation causes acute inflammation as erythema and subsequent pigmentation.

Extracellular reactive oxygen species generated by UV induce the release of arachidonic acid from le cell membranes *via* the enzymatic action of phospholipase A2. Arachidonic acid is then transformed into prostaglandins and leukotrienes by cyclooxygenase and lipoxygenase respectively.

The anti-inflammation properties of LIGHTOCEANE® are shown by the help of *in tubo* assay and *in vitro* test on reconstructed skins.

Inhibition of 5-lipoxygenase

In tubo test

The 5-lipoxygenase generates hydroperoxydes which are the precursors of leukotrienes.

Leukotrienes have potent biological actions, such as degranulation and plasma exudation (Samuelsson *& al.*, 1987 – Science, 237 : 1171-1176). They also participate in host defence reactions and pathological conditions, such as immediate hypersensitivity and inflammation.

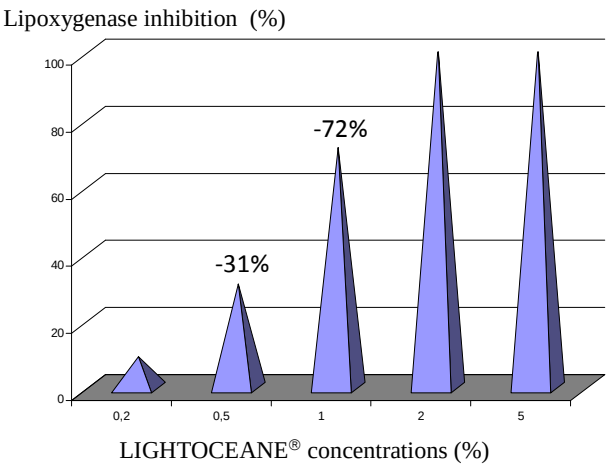
Methods

This method uses an insaturated fatty acid (the linoleic acid) as the substrate.
In presence of the enzyme, there is formation of a peroxide (5 HPETE : 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid). The reaction is followed at 234 nm.

Results

Results are expressed in % of anti-5-lipoxygenase activity comparatively to control realized without LIGHTOCEANE®.

They represent the average of three experiments for each concentration tested (α= 0.05).



➤ LIGHTOCEANE® inhibits the production of 5-lipoxygenase

Note : 72% inhibition with 1% active.

Inhibition of IL 1α

IL1α is involved in various immune responses and inflammatory processes.

It is suspected to be involved in the genesis of age spots (Okazaki , M *& al*., 205 – Br.J. Dermatol.153 (suppl.3) 23-29).

In vitro study on reconstructed skins

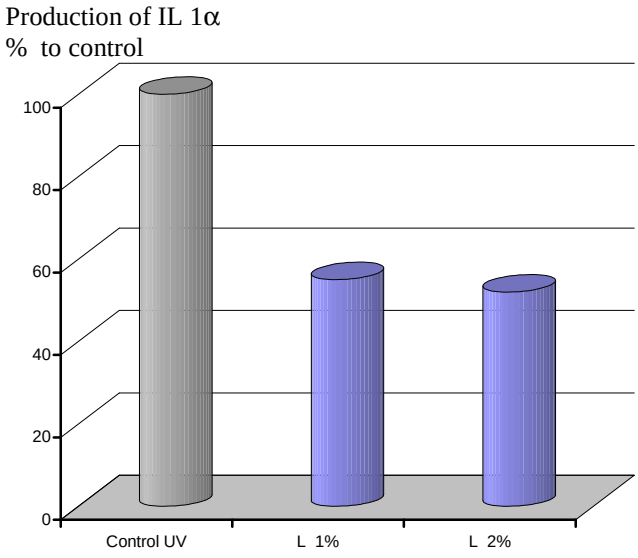
The aim of this study is to evaluate the anti-irritant potential of LIGHTOCEANE® by using an IL1α assay.

Methods

The release of IL 1α is measured with an Elisa kit on reconstituted skin submitted to UVB (300 mJ.cm²) after 72 h cultivation in the presence of 1% and 2% active.

Results

This graph demonstrates that LIGHTOCEANE® inhibits the IL 1α production in reconstructed skins submitted to UVB irradiation.



At 1 % LIGHTOCEANE® induces an inhibition of 45 % compared to irradiated control.

At 2 % LIGHTOCEANE® the inhibition equals 48 % .

➤ LIGHTOCEANE® inhibits the production of inflammation mediators that also stimulate melanogenesis.

➤LIGHTOCEANE® shows anti-inflammation activity in the both assays, as many skin lightening ingredients which are under current use have skin irritating potential.

➤As IL 1α is suspected to be involved in the formation of age spots, LIGHTOCEANE® will be useful for the prevention of age spots.

LIGHTOCEANE® boosts the skin metabolism

LIGHTOCEANE® shows also additional properties useful to fight skin ageing. It improves cell metabolism and inhibits both elastase and collagenase.

LIGHTOCEANE® is able to boost cell metabolism.

Increase of cell proliferation

In vitro study on human keratinocytes

Methods

Human keratinocytes are seeded at 8000 cells per well (96-well microtiter plate) in an optimal medium for 24 h before addition of the active. LIGHTOCEANE® is tested at 4 concentrations : 0.5 -1 -1.5 & 2 %).
After 48 h incubation, the cell proliferation is determined by the MTT test.

Results

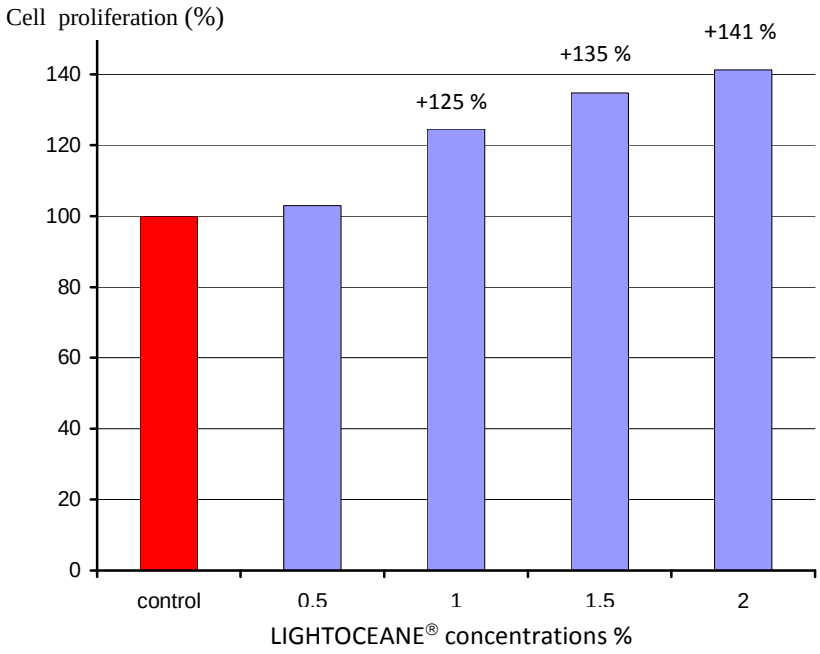
Results are expressed as percentage against control (without ingredient). They represent the average of 2 experiments with 4 values for control and each concentration.

Results are validated statistically by two methods :

- the analysis of variance ANOVA (fix model) with
  $\upsilon 1=4$ and $\upsilon 2=20$
  α : 0.001
  Fα : 14.819
  Fc : 50.795 ***

- the least significant difference with
  $\Delta c = | m1-m2|$ and
  $\Delta = t\alpha V [(2. \upsilon i)/2]$
  υI : intrinsic variance.

  Control=0.5 % < <1 %
  < 1.5 % = 2 %.



➤ With 1 % LIGHTOCEANE®, the cell proliferation increases of 25 % compared to control without any active.

LIGHTOCEANE® protects the dermal fibers against proteases deterioration

Proteases produce a decay of the proteins which structure the extracellular matrix of the inner skin. LIGHTOCEANE® is able to inhibit both collagenase, elastase and hyalyronidase.

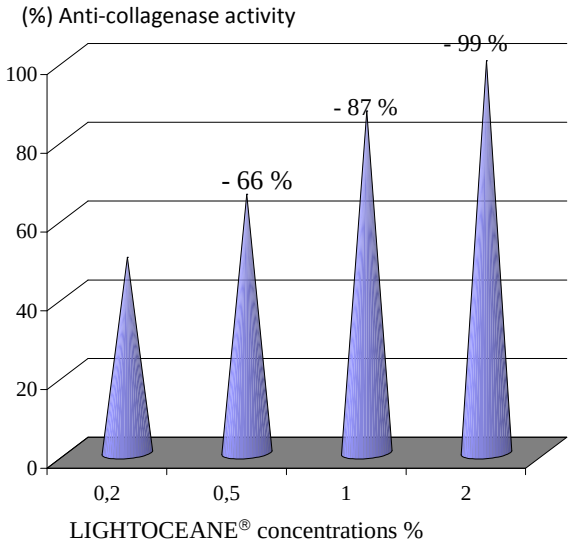
Anti-collagenase activity

Method

The tests are carried out with a *Clostridium* collagenase and a synthetic chromogenic substrate FALGPA.

Results

Results represent the average of 3 values for control and each concentration ($\alpha = 0.05$).



➤ LIGHTOCEANE® inhibits the activity of collagenase

Note 87 % inhibition with 1 % active .

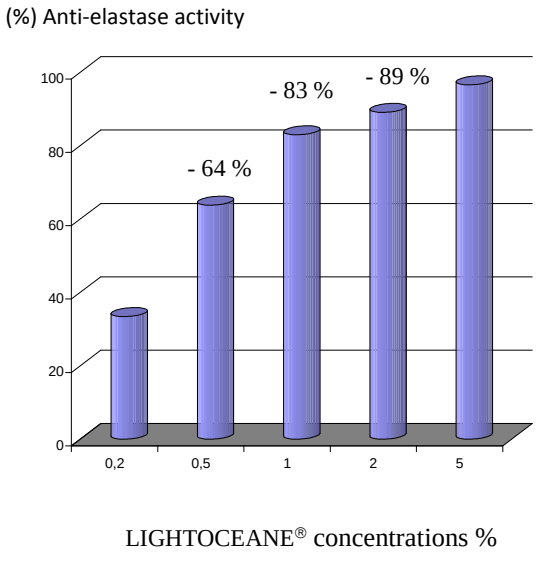
Anti-elastase activity

Method

Porcine pancreatic elastase is assayed spectrophotometrically using [N-succ-(Ala) 3-p-nitroanilide] as the substrate and monitoring the release of p-nitroaniline for 20 min at 25°C.

Results

Results represent the average of 3 values for control and each concentration tested ($\alpha = 0.05$).



➤ LIGHTOCEANE® inhibits the activity of elastase

Note 83 % inhibition with 1 % active.

➤ The action of LIGHTOCEANE® is found to be dose-dependent for both collagenase and elastase.

Anti-hyaluronidase activity

Hyaluronidases are endoglucoaminidases which degrade hyaluronan and to a lesser extent other glycosaminoglycans.

The hyaluronic acid constitutes one of the most important structural and functional elements of numerous tissues. It acts as a “true molecular sponge” because it can absorb a large quantity of water. Its turn over is very high for 24 hours, due to hyaluronidase hydrolysis. It is important in controlling tissue hydration (Block & Bettelheim, 1970 – Biochem. Biophys. Acta, 201:69).

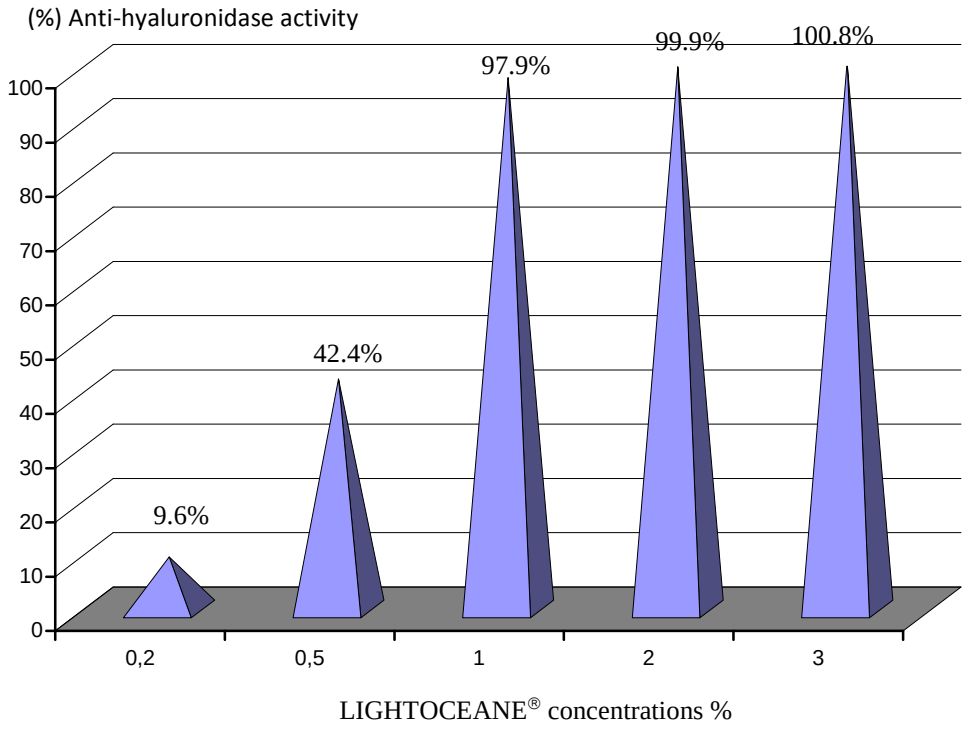
During aging, its quantity decreases, modifying the viscoelasticity of the skin. Consequently, it is necessary to prevent hyaluronic acid hydrolysis.

Method

Hyaluronidase activity is determined spectrophotometrically according to the method of Reissig *& al.*, (1955- J. Biol. Chem., 217:959-969).

Results

Results represent the average of three experiments for control and each concentration tested ($\alpha = 0.05$). They are expressed in % of anti-hyaluronidase activity in presence of 5 concentrations in LIGHTOCEANE® (0.2– 0.5 – 1 – 2 and 3%) compared to control without algal extract.



➤ LIGHTOCEANE® shows a dose-dependent anti-hyaluronidase activity.

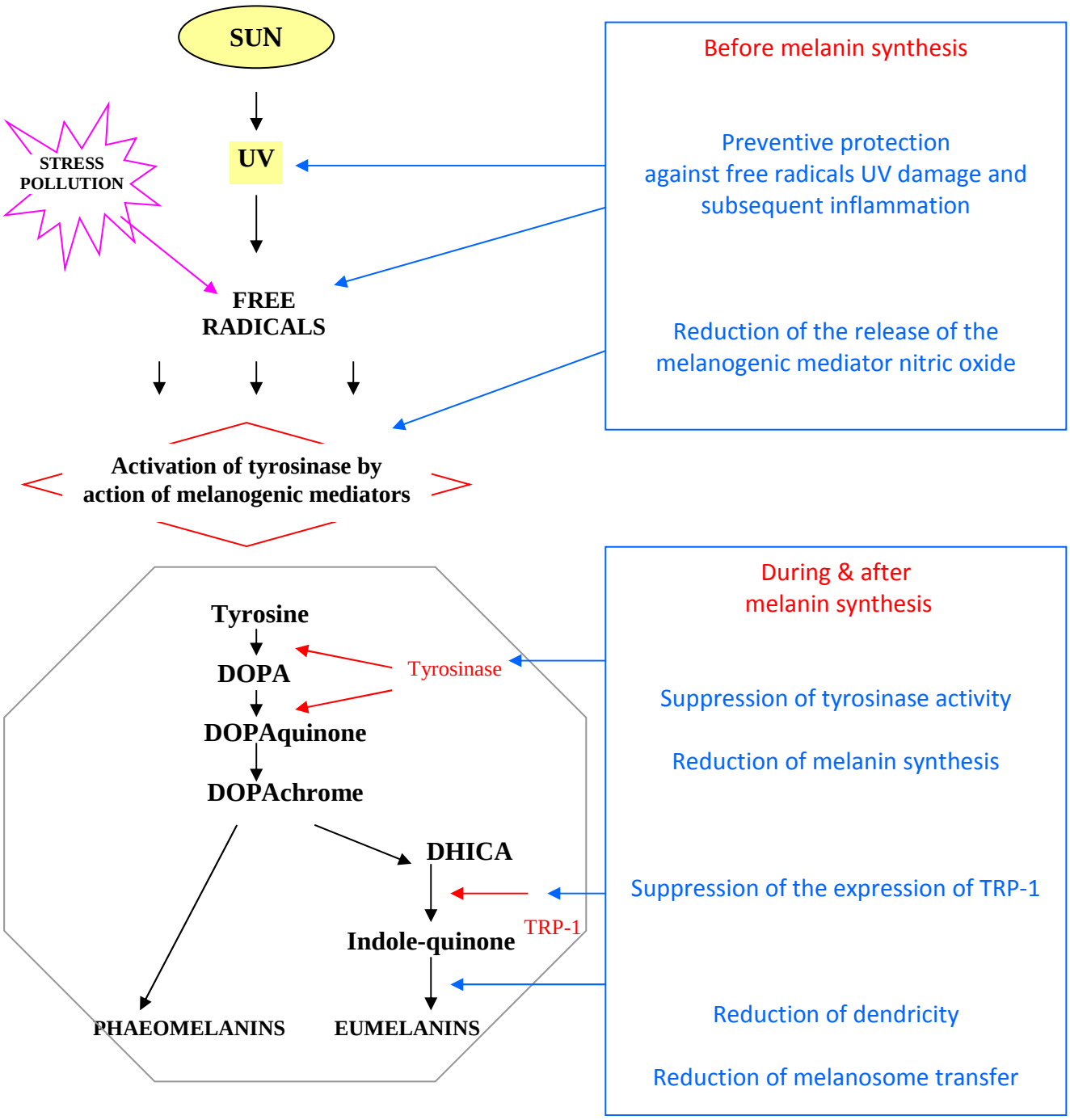
Note more than 97% inhibition with 1 % active .

CONCLUSION & COSMETIC BENEFITS

Developing a concept based on innovation, effectiveness and tolerance LIGHTOCEANE® proposes a complete and innovative mode of action to reduce skin pigmentation.

Its depigmenting effectiveness has been proven i*n vitro* and *ex vivo*.

LIGHTOCEANE acts with a clever mechanism of action at all levels of the pigmentation cascade.



LIGHTOCEANE® is a double inhibitor of the pathway of melanin synthesis. It inhibits tyrosinase by reducing the quantity of melanin. It inhibits TRP-1 implied in the final steps of the synthesis of eumelanins. So it decreases the production of black melanins.

What are the other advantages of LIGHTOCEANE® ?

- natural and marine origin
- safe, no cytotoxic, no mutagenic
- hypoallergenic
- anti-oxidant
- protection against both UVB & UVA
- anti-inflammatory and anti-irritant
- anti-elastase, anti-collagenase and anti-hyaluronidase.

These additional properties, protective and preventive, make LIGHTOCEANE®more attractive as an active in cosmetic products.

The functions of LIGHTOCEANE® may be derived from polyphenols compounds.

COSMETIC APPLICATIONS

Skin care:

- Lightening gels and emulsions skin care products
- Lightening ranges for body care products (hand, forearms)
- Anti-age skin care
- Anti-age spot creams & serums.

Recommended use levels: 1 % - 3 %.

OTHER VERSIONS OF LIGHTOCEANE®

Version LIGHTOCEANE EL

Appearance: liquid limpid brown coloured with possible precipitates

INCI names

water	CAS n° 7732-18-5	EINECS n°231-791-2
algae extract	CAS n° 92128-82-068917-51-1	EINECS n° 295-780-4/-

CHINA LISTED 2014

algae extract

Preservatives by selection: microcare SB or phenoxyethanol.

Version LIGHTOCEANE G 40

Appearance: liquid limpid brown coloured none precipitates

INCI names

glycerin	CAS n° 56-81-5	EINECS n°200-289-5
water	CAS n° 7732-18-5	EINECS n°231-791-2
Halidrys siliquosa extract		

Preservatives by selection: microcare SB or phenoxyethanol.

Version LIGHTOCEANE G 40 - EL

Appearance: liquid limpid brown coloured none precipitates

INCI names

glycerin	CAS n° 56-81-5	EINECS n°200-289-5
water	CAS n° 7732-18-5	EINECS n°231-791-2
algae extract	CAS n° 92128-82-068917-51-1	EINECS n° 295-780-4/-

CHINA LISTED 2014

algae extract

Preservatives by selection: microcare SB or phenoxyethanol.

ANNEX

Evaluation of ocular irritation

eurofins | ATS

N°Etude : 301719F01
Version : 01
Page 1 sur 13
P04.3.DPL.00014.01

RAPPORT D'ETUDE

GELYMA
1 boulevard de l'Océan
Parc d'Affaires Marseille
Batiment C 4
13009 MARSEILLE

Le 18/12/2008

EVALUATION DU POTENTIEL IRRITANT D'UN PRODUIT PAR APPLICATION SUR LA MEMBRANE CHORIO-ALLANTOÏDIENNE DE L'ŒUF DE POULE :
Méthode du Het Cam

SUMMARY

The Het Cam test is performed on the chorioallantoic membrane of an embryonic chicken egg after eleven days of incubation. This membrane has the characteristic to be vascularized.

The principle of this study is based on the visual observation, by a trained person, of the possible irritations (hyperaemia, haemorrhaging, coagulation / thrombosis) that may appear during the five minutes that follow the application of the said product on this chorioallantoic membrane.

This study has been carried out following the official method of the Official Journal of French Republic, according to the order of april 5th, 1971, modified in the last by the order of november 29th, 1996.

According to the performed experimental conditions, **the product LIGHTOCEANE SANS CONSERVATEUR, referenced GELYMA 08**, tested by the HET CAM method, at 5 %, can be considered as **slightly irritant** regarding its ocular primary tolerance.

Eurofins ATS -Pôle d'activité d'Aix-Les Milles - Actimart - 1140, Rue Ampère - 13851 Aix-en-Provence Cedex 3 - France
TEL +33 (0)4.42.39.78.08 - FAX +33 (0)4.42.39.77.81
N° SIRET : 33761796300083 – Code APE : 7120 B

Evaluation of cutaneous irritation

eurofins | ATS

N° Etude : 301720F01
Version : N° 1
Page : 13/15 + annexe 2
P05.0.DOC.00017.02

STUDY SUMMARY

EVALUATION OF SKIN TOLERANCE OF A COSMETIC PRODUCT AFTER A SINGLE APPLICATION UNDER OCCLUDED PATCH DURING 48H ON 13 VOLUNTEERS:
48 hours occluded patch tests

- **Product tested**: LIGHTOCEANE SANS CONSERVATEUR
- **Promotor**: GELYMA
- **Objective**: Assessment of the cutaneous local tolerance of the studied product after an epicutaneous test performed in occluded conditions, during 48 hours.
- **Place of the study:** EUROFINS ATS
  Pôle d'activité Aix-Les-Milles - ACTIMART
  3 allée des Ingénieurs
  1140 rue André Ampère
  13851 AIX EN PROVENCE cedex 3
- **Investigator**: Doctor Mary Crest
- **Dates of study:** from 02/12/08 to 04/12/08
- **Method:**

✓ ***Application:***

Area: on the back
Quantity of product: 0.02 ml
Frequency and duration: only one application during 48 hours
Conditions of application: product applied diluted at 5% under occluded patch.

✓ ***Assessment method:***

A dermatologist performs the clinical observation, after the removal of the patches. The quantification of the cutaneous irritation is given through a numeric scale (erythema, oedema, dryness/desquamation, vesicles). The average irritant score of the product to be tested is calculated from the average of the quotations obtained for each volunteer, allowing to rank the product from "non irritant to very irritant". The assessment is always made by comparison with the "negative" control.

- **Panel:** 13 healthy adult volunteers.
- **Result:** The average irritant score of the product is 0,00.
- **Conclusion:**

According to the experimental conditions of the study, **the product LIGHTOCEANE SANS CONSERVATEUR, referenced Lot 08 10 180**, can be considered as **not irritant regarding its primary cutaneous tolerance.**

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N° SIRET : 33761796300083 – Code APE :7120B

Evaluation of mutagenicity

vivotecnia

Final Report B-00746
Rapport final B-00746

BACTERIAL REVERSE MUTATION TEST

B-00746 FINAL REPORT

LIGHTOCEANE

BATCH: **08 10 180**

The present bacterial reverse mutation test (Ames test) was performed in order to evaluate the mutagenic potential of the test item.

The test was performed in accordance with OECD Guideline 471 for the Testing of Chemicals (Bacterial Reverse Mutation Test. Adopted 21st July 1997) and the test Method B13/B14 of Commission Directive 2000/32/EC.

Doses ranging from 5µL to 0,06µL per plate were tested. No cytotoxicity was observed at any dose.

Suspensions of 5 amino-acid requiring strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535, TA1537) were exposed by the direct plate incorporation method to five doses of the test item in the presence and in the absence of an exogenous metabolic activation system. Both tests were repeated with the pre-incubation method.

Revertant bacteria due to point or frameshift-mutations at specific locus are able to grow, forming colonies. These colonies were counted and compared to the number of spontaneous revertant colonies on solvent control plate (negative control). Similarly, specific standard mutagens were tested and used as positive controls.

Based on the results obtained in this study, the test item **LIGHTOCEANE** was found to be NON MUTAGENIC and NON-PROMUTAGENIC under the test conditions.

Test facility
VIVOTECNIA Research S.L.
Parque Científico de Madrid
C/Santiago Grisolía, 2
28760 Tres Cantos (Madrid)
Spain

Evaluation of sensitizing potential

ROBEN PRODUCTION GRUP SRL

CENTRUL DE CERCETARE A PLANTELOR
STRADA LUGOJ NR. 63 SECTOR 1, BUCURESTI, ROMANIA

EVALUATION DU POUVOIR SENSIBILISANT CHEZ LE VOLONTAIRE ADULTE SELON LA METHODE DE MARZULLI-MAIBACH

ASSESSMENT OF SENSITIZING POTENTIAL IN THE ADULT VOLUNTEER FOLLOWING THE METHOD OF MARZULLI-MAIBACH

Etude clinique sur 107 volontaires, tout type de peau
Clinical study on 107 volunteers, with all skin type

- Etude/ *Study*: 3.04
- Produit/ *Product*: RB11/0006

PRODUIT / *Product*	**: LIGHTOCEANE SANS CONSERVATEUR**
CODE PRODUIT / *Code product*	**: RB11/0006**
DILUTION / *Dilution*	**: PUR** **PURE**
INVESTIGATEUR / *Investigator*	**: DR. ANNE-MARIE MARINESCU**

CONCLUSIONS/ *CONCLUSIONS*

Dans les conditions d'une application répétée de la procédure de patch-test conduite auprès d'un panel de 107 volontaires présentant tout type de peau, le produit **LIGHTOCEANE SANS CONSERVATEUR, RB11/0006** a été «Testé dermatologiquement» et n'a pas présenté de risque d'irritation de la peau cliniquement significative ni montrer de réaction de type allergique au contact de la peau humaine.

Under the conditions of a repeated insult (occlusive) patch test procedure conducted in a panel of 105 subjects, with all skin type, the product **LIGHTOCEANE SANS CONSERVATEUR, RB11/0006** *was "Dermatologist-Tested" and did not induce clinically significant skin irritation nor show any evidence of induced allergic contact dermatitis in human subjects.*

Le produit **LIGHTOCEANE SANS CONSERVATEUR, RB11/0006** peut être considéré comme «hypoallergénique».

The product **LIGHTOCEANE SANS CONSERVATEUR, RB11/0006** *can be considered as "hypoallergenic".*

Parc d'Affaires Marseille Sud
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13009 Marseille –France

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Contact

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e-mail gelyma @ wanadoo.fr