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(54) Title: PRODUCTION METHOD OF MERISTEMATIC CELLS OF PLANTAGO LANCEOLATA, COMPOSITION COMPRISING SAID CELLS OR THEIR CELLULAR EXTRACT, AND COSMETIC, NUTRACEUTICAL AND DERMATOLOGICAL USES

(57) Abstract: According to the invention, meristematic cells are obtained from a selected cell line and mainly comprise plantamajosiide. They can be advantageously used in cosmetics, especially for anti-aging nutraceuticals or dermatological applications



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**PRODUCTION METHOD OF MERISTEMATIC CELLS OF PLANTAGO LANCEOLATA,
COMPOSITION COMPRISING SAID CELLS OR THEIR CELLULAR EXTRACT,
AND COSMETIC, NUTRACEUTICAL AND DERMATOLOGICAL USES**

5 TECHNICAL FIELD

The present invention is directed to meristematic cells from a new cell line of *Plantago lanceolata*, cells thus obtained, an extract of said cells, compositions comprising them and uses in the fields of cosmetics, nutraceutics and dermatology.

More specifically, the present invention is directed to the cosmetics and personal care product industry applied to skin and appendages (such as body hair, eyelashes, eyebrows, nails, hair) of mammals, human or animal.

BACKGROUND ART

Plantago lanceolata is an herbaceous perennial plant of the Plantaginaceae family that grows in all temperate climates of Europe and north and central Asia. It is also called plantain, ribwort plantain, ribgrass, ripple-grass, English plantain, buckhorn plantain, narrowleaf plantain, lanceleaf plantain, ribleaf and lamb's tongue.

It has always been known for its medicinal properties: antitussive, anti-inflammatory, anti-irritant, bechic, expectorant, healing, external antiseptic and antibiotic. These properties can be attributed to the different compounds synthesized by the plant and that can be extracted from the different parts of the plant, and according to the extraction method and solvent used. It was listed that in *Plantago lanceolata* iridoidic heterosides, phenylpropanoid glycosides, mucilage, flavonoids, phenolic acids, tannins, saponosids and essential oils could be found in particular.

The extracts can be obtained by usual extraction methods directly from plant parts or by *in vitro* methods of plant culture of meristematic cells (or undifferentiated cells). *In vitro* methods have advantages including to overcome the fluctuations related to culture conditions and to reproducibly stimulate production by plant cells of secondary metabolites of particular interests, which otherwise have low yields, as it is the case for phenylpropanoid glycosides for example. Profile and quantities of bioactive compounds present in the cells obtained by *in vitro* methods will be different from those obtained by other extraction techniques, the dedifferentiated cells being not capable of producing all secondary metabolites and the cultures being advantageously oriented towards the production of selected secondary metabolites. A cell extract or meristematic cell culture will therefore not necessarily have at the end the same activities that another type of extract, in particular compared to a plant extract not realized by cell culture.

These *in vitro* methods include schematically:

- 35 - In a first step, where appropriate, the establishment of cell lines from callus (cluster of undifferentiated cells) obtained on cuts of plant parts (leaf, root, stem...);

- Selection of a cell line capable of large-scale producing a biomass of meristematic cells according to pre-established criteria (constant phenotype and optimal and consistent production of selected metabolites, ability to proliferate);

- Then from this selected line: generating said cell biomass, possibly with an elicitation phase; and finally

- In a third step, treatment of obtained cell biomass to recover the meristematic whole cells and if necessary extraction of the content of the cells.

It is these extracts and/or whole cells or partially crushed cells that will then be used in cosmetic, dermatological and/or nutraceutical compositions.

Patent application EP2319914 discloses this technique for obtaining meristematic cells with a high yield in caffeic acid derivatives for a theoretical list of 33 plant species including *Plantago lanceolata*. The referred caffeic acid derivatives include phenylpropanoid glycosides and caffeoylquinic acids. Test results are given only for 5 species and each one for a different activity: *Buddleja davidii* specie (antioxidant activity), *Leontopodium alpinum* specie (hyaluronidase inhibitory activity), *Lippia citriodora* specie (anti-inflammatory), *Gardenia jasminoides* specie (collagenase inhibitory activity) and *Echinacea angustifolia* specie (collagen stimulation activity).

Phenylpropanoid glycoside properties have been widely disclosed, mainly antioxidant and anti-inflammatory properties that can be valorized in the areas of cosmetics, dermatology and nutraceuticals.

SUMMARY OF THE INVENTION

The aim of the present invention is to propose a vegetable biological material from a plant of the *Plantago lanceolata* specie for industrially obtaining, that is to say in a reproducible manner, by an *in vitro* cell culture a composition having remarkable activities, in particular in relation to the presence of phenylpropanoid glycosides.

To this end, the present invention proposes a method of *in vitro* producing of *Plantago lanceolata* meristematic cells comprising phenylpropanoid glycosides from a stabilized cell line, characterized in that the cell line is the selected IRB PL3 cell line.

This cell line has been deposited at DSMZ (Deutsche Sammlung von Mikroorganismen Zellkulturen GmbH Inhoffenstraße 7B, 38124 Braunschweig, Germany) by the applicant IRB under the provisional reference: *Plantago lanceolata* IRB PL3.

Meristematic cells thus obtained are rich in phenylpropanoid glycosides, mainly in plantamajoside. The proportion by weight of total glycosides phenylpropanoid in the lyophilized biomass, evaluated by UV spectrometry at 330 nm, expressed as plantamajoside equivalent, is about 10%. Proteins, amino acids, lipids, and polysaccharides have also been identified as classes of compounds in the cells of the invention.

The selection and use of the IRB PL3 *Plantago lanceolata* cell line provides meristematic cells producing advantageously unexpected and remarkable results at all levels of skin: dermis,

dermal/epidermal junction (DEJ) and epidermis, as well as remarkable anti-inflammatory, anti-oxidant and anti-glycation results, thereby contemplating applications in the areas covered by the invention. It was also demonstrated that the products obtained with cells according to the invention advantageously act on a number of micro-RNAs. Micro-RNAs are produced by the cell from DNA, such as mRNA, and play a crucial role in controlling many physiological processes by inhibiting the synthesis of specific proteins. The results on micro-RNAs clearly reinforce the intended applications and open to other perspectives.

In vivo, applying the product according to the invention improves dermis quality, which appears both firmer, elastic (cutometry) and thicker (ultrasound), brings a beneficial effect on senescence spots (analysis by pictures of skin clarity with ColorskinTM software), a thickening of the epidermis (laser confocal microscopy/VivascopeTM), a more dense skin (ReviscometerTM) and a soothing of the skin surface (imprint scanning and analysis images). 

Thanks to the observed effects with the product according to the invention, both *in vitro* and *in vivo*, the product can advantageously be positioned as a global anti-age.

More broadly, the present invention also provides the use of *Plantago lanceolata* cells to densify dermis, strengthen dermal-epidermal junction, thicken epidermis and/or provide anti-glycation defense means to skin.

The results are given below in the description.

The method according to the invention can be achieved according to the following steps:

- From the IRB PL3 *Plantago lanceolata* line producing a critical pre-biomass by successive pre-cultures and increasing sizes;
- Produce a biomass of meristematic cells in a bioreactor using said pre-biomass and a suitable culture medium; and
- Separating said biomass enriched in phenylpropanoid glycosides from said culture medium and recovering said meristematic cells.

According to further features of the invention:

- The bioreactor production stage comprises an elicitation step, thus advantageously allowing increasing the content of plantamajoside and more generally in phenylpropanoid glycosides (PPG); and/or

- The biomass from the reactor is collected by filtration after a culture period of 7 to 21 days, preferably between 10 and 14 days, thus advantageously producing the greatest quantity of biomass, with high viability; and/or

- An additional step of drying the meristematic cell biomass separated from the culture medium, thus advantageously testing their biological activity and their long-term storage, without having to add any preservatives.

Elicitation of compounds of interest can generally be done by adding microbial fractions to the culture (for example *saccharomyces* yeasts), adding biological molecules to the culture such as chitosan,

methyljasmonate, jasmonic acid or salicylic acid, adding non-biological molecules to the culture such as paclobutrazol, application to the culture of a variation of temperature, pH or an osmotic stress induced by a non-metabolizable sugar, such as mannitol, the use of a more drastic depletion of macroelements and sugar in the medium, and/or adding to the culture adsorbent resins which, besides
5 eliciting production of interest compounds, can trap them.

Preferably according to the invention, elicitation is performed by changing the culture medium, including the nutrient levels.

The subject matter of the present invention is also the selected IRB PL3 *Plantago lanceolata* line, meristematic cells of *Plantago lanceolata* obtained through the use of this line, the invention method
10 disclosed above and a cellular extract obtained from said cells, the extract being for example made by cell lysis, then a centrifugation step followed by filtration, so as to recover the inside of the cells and to eliminate cell walls.

The present invention also provides a composition comprising *Plantago lanceolata* meristematic cells or a cell extract according to the invention in a physiologically acceptable medium and cosmetic,
15 nutraceutical or dermatological uses of said composition, of said cells or said cellular extract.

Cosmetic uses according to the invention

MicroRNAs (or miRNAs) were discovered in 1993. Since then, the role of microRNAs has been pinpointed and seems to have expanded over the years. MicroRNAs are numbered in the order in which they were discovered, forming a different class of very small (20 to 25 nucleotides long), non-
20 coding RNAs. MicroRNAs are produced by cellular DNA, just like mRNA, and play a key role in controlling numerous physiological processes by inhibiting specifically the synthesis of various proteins. Once produced, the microRNA specifically binds to the beginning of its own mRNA, thereby rendering the mRNA information impossible for the ribosome to read or turning it into the target of proteins that will destroy them. These post-transcriptional control phenomena are real natural on-off
25 switches. They may affect at least 30% of gene production. Some miRNAs, repressor of collagen I, collagen IV and elastin synthesis were identified. Some other miRNAs determine the appearance of senescent phenotypes, in particular in fibroblasts.

Expression modulation of these switches is thus an advantageous way in an anti-aging strategy to boost certain protein synthesis and slow cellular senescence.

30 With age, skin changes. It becomes thinner and loses density. It sags and loses firmness. It also becomes spotted. Pigmented areas appear and then expand to form what is commonly known as "age spots" on the hands, face and bust. To varying degrees, our lifestyle exacerbates the normal skin ageing process: sun exposure, tobacco use, pollution and poor eating habits can accelerate the chronological ageing process of the body's organs, including the skin. Underlying these macroscopic

changes is disorganization at the tissue and cellular levels. Aging is characterized by a succession of "drifts" with regard to a previous state which allowed having youthful skin (homeostasis).

As a result, the loss of density and thinning of the dermis are related to a reduction in the synthesis of macromolecules (in particular collagen I, collagen IV, elastin and hyaluronic acid) by dermal fibroblasts, the cells in charge of producing them. A simultaneous increase in matrix metalloproteinase (MMP) production that is concomitant with the decrease in the production of TIMP (Tissue Inhibitor of Metalloproteinase), the natural inhibitors of MMP, leads to the fragmentation and further degradation of the aforementioned macromolecules.

The increase in MMP degradation activity and the reduction of protein synthesis also affects the dermal-epidermal junction (DEJ), where key skin proteins are found. Collagen IV is one of these key proteins. It is organized into a thin layer to which other structures essential for skin homeostasis, like laminins, attach. With age, collagen IV becomes more fragmented and its production slows, just like for laminins. In certain areas, this leads to changes in the relationships between melanocytes, keratinocytes and the DEJ. The result is an increase in skin pigmentation, including the appearance of age spots or senile lentigo in these areas.

Epidermis also undergoes the effects of aging. Cell renewal is less important and therefore the skin becomes thinner. Furthermore, the connections between the cells are less strong. Filaggrin and hyaluronic acid are less synthesized, causing a lesser intercellular cohesion and higher water loss in the *stratum corneum*.

Finally, increased level of pre-inflammatory mediators (prostaglandins and interleukins types) whose presence is often associated with aging, and increased level of many pro-oxidant compounds that will increase all the deleterious effects already described by oxidizing and thus degrading/inactivating the molecules that contribute to cellular homeostasis (lipids, proteins, sugars, nucleic acids), are observed. A particular phenomenon, the glycation of proteins, enhances aging by modifying all skin proteins (structural or functional), and in particular dermal proteins such as collagen, disorganizing the extracellular matrix and making it lose its tonicity, its flexibility. This glycation will generate an increase in radical products which in turn will disrupt the metabolisms and degrade cell structures of the skin.

The meristematic cells, the cellular extract or a composition comprising them according to the invention, act advantageously in five different and complementary directions of skin aging as discussed above.

1) Dermal densification

- Decrease of the expression level of three known miRNA to slow down the synthesis of collagen I (miRNA-29 and miRNA-196a family), elastin (miRNA-29 family) and TIMP-1 (miRNA-21); and

- Stimulation of the synthesis of dermal macromolecules (collagen I, elastin and hyaluronic acid in particular), TIMP, which inhibit MMPs.

Thanks to the invention, the dermis keeps its density, hydration, will recover elasticity and firmness; the face will gain a smooth relief (reduction of wrinkle depth).

5 **2) Maintaining the JDE integrity and thereby anchoring keratinocytes and melanocytes control**

- Decrease of the expression level of miRNA-29 known to slow down the collagen IV synthesis and of miRNA-21 which inhibit the TIMP-1 synthesis.

10 Thus the synthesis of collagen IV and laminins are increased, thereby reinforcing the JDE, the relationships of these components with the basal layer of keratinocytes and allowing observing a lightening of age spots.

3) Reinitiate epidermal metabolisms to thicken the epidermis

- Accentuate keratinocyte differentiation,
- Stimulates the production of two proteins (claudin-1 and Zo-1) present at the level of tight
15 junctions, which ensure the cohesion between the corneocytes, and
- Stimulates the synthesis of hyaluronic acid and filaggrin, which will promote proper hydration of the skin.

The *in vivo* result of these *in vitro* effects is a thickening of the dermis.

4) Decrease in pre-inflammatory messengers

- 20 - Decrease of the three pre-inflammatory messengers (PGE2, IL6 and IL8) on human keratinocytes.

The presence of these messengers affects cell aging, in particular PGE2 involved on melanocytes by increasing tyrosinase activity, their dendricity and therefore the transfer of melanin to keratinocytes, which produces hyperpigmentations.

25 **5) Generic anti-senescence signals; antioxidant action and miRNA involved in aging.**

- Antiradical power;
- Protective activity against lipid peroxidation induced by UVA;
- Decrease in the basal rate of reactive oxygen species in human dermal fibroblasts;
- Induction on this same cell type of overproduction of superoxide dismutase, a decisive
30 enzyme for fighting oxidation;
- Protection against protein glycation; and
- Reduction of the expression of miRNA-30a, 34a, 181a and -152 on fibroblasts. This effect is connected to an extension of cell survival and a reduction of the appearance of senescent phenotype (on fibroblasts).

35 The anti-aging activity on dermal and epidermal extracellular matrix are not described on Plantago extracts of prior art.

The results demonstrating the activity of the product according to the invention are described below in details.

Moreover, comparative tests with only verbascoside were performed and showed that the meristematic cells of the invention result in superior effects on the synthesis of dermal macromolecules (collagen I and hyaluronic acid), the synthesis of molecules responsible for the DEJ strengthening (laminins) and the strengthening of the epidermal barrier (hyaluronic acid). In addition, meristematic cells according to the invention showed a protective effect of cells toward a cytotoxic effect demonstrated contrariwise with verbascoside.

Superior effects with cells according to the invention in comparison with the pure verbascoside which is the most notorious representative of this class of phenylpropanoid glycoside molecules are therefore observed.

Dermatological applications according to the invention

Topical application of meristematic cells, of the cellular extract or of a composition containing them, according to the invention (or a composition containing the meristematic cells or their extracts according to the invention), for the treatment pathologies of the skin chosen among:

- Pigmentation disorders;
- Disorders of an inflammatory nature (such as atopic dermatitis, psoriasis, acne);
- Bullous dermatoses;
- The diseases involving disorders in the synthesis of collagen, elastin, hyaluronic acid, and/or
- Dry skin, rosacea and ichthyosis.

Nutraceutical applications according to the invention

Orally (ingestion), to obtain a beneficial effect to strengthen the intestinal wall. For example, possible absorption of 10 to 50mg of lyophilized cells per day and per person.

Composition preparation

The present invention therefore proposes a composition, especially topical, comprising meristematic cells, or an extract of said cells according to the invention in a physiologically acceptable medium. According to the excipient and to the dosage of the cells or cellular extract, this composition will be for example a concentrated active ingredient or a less concentrated final composition directly intended to the end user.

“Physiologically acceptable medium” means according to the present invention, without limitation, an aqueous or hydroalcoholic solution, a water-in-oil emulsion, an oil-in-water emulsion, a micro-emulsion, an aqueous gel, an anhydrous gel, a serum, a dispersion of vesicles, or a powder.

“Physiologically acceptable” means that the compositions are suitable for topical or oral use, in contact with mucous membranes, appendages (nails, hair and body hair), scalp and skin of mammals, particularly human, compositions which may be ingested or injected into the skin, without risk of

toxicity, incompatibility, instability, allergic response, and others. This “physiologically acceptable medium” forms what is commonly called the excipient of the composition.

The meristematic cells and/or the cellular extract of these cells may be combined with other active ingredients in effective concentrations to act synergistically or to reinforce and to achieve the desired effects described in the invention, such as the following ingredients: radiation filters, including UVA and/or UVB, moisturizing, calming, muscle relaxant, slimming, restructuring, firming, re-filling, tensing, acting on microcirculation, acting on inflammation, on free radicals, vitamins, anti-wrinkle lightening agents, etc.

The composition according to the invention can be applied to the face, body, neck, neckline, scalp, hair, eyelashes, body hair, in any form or vehicles known from the ones skilled in the art, in particular in the form of solution, dispersion, emulsion, paste or powder, individually or as a premix in vectors such as macrocapsules, microcapsules or nanocapsules, macrospheres, microspheres or nanospheres, liposomes, oleosomes or chylomicrons, macroparticules, microparticules or nanoparticules, macrosponges, microsponges or nanosponges, microemulsions or nanoemulsions, or adsorbed on organic polymer powders, talcs, bentonites, spores or exines and other inorganic or organic supports.

In cosmetics for example, applications can be proposed in particular in the ranges of skin care products for face, body, hair and body hair and makeup-care lines, including eyebrows and eyelashes.

The cells or the cellular extract of the present invention may in general be used in any form whatsoever, in a form bound to or incorporated in or absorbed in or adsorbed on macro-, micro-, and nanoparticles, or macro-, micro-, and nanocapsules, for the treatment of textiles, natural or synthetic fibers, wools, and any materials that may be used for clothing or underwear for day or night intended to come into contact with the skin, handkerchiefs or cloths, to exert their cosmetic or therapeutical effect via this skin/textile contact and to permit continuous topical delivery.

The CTFA International cosmetic ingredient dictionary & handbook (13th Ed. 2010) (published by the Cosmetic, Toiletry, and Fragrance Combination, Inc., Washington, D.C.) describes a non-limited wide variety of cosmetic and pharmaceutical ingredients conventionally used in the skin care industry that can be used as additional ingredients/compounds in the compositions of the present invention.

Further skin care and hair care active ingredients that are particularly useful can be found in Sederma's commercial literature and on the website www.sederma.fr.

The following commercial actives can also be mentioned, as examples: betain, glycerol, Actimoist Bio 2™ (Active organics), AquaCacteen™ (Mibelle AG Cosmetics), Aquaphyline™ (Silab), AquaregulK™ (Solabia), Carciline™ (Greentech), Codiavelane™ (Biotech Marine), Dermaflux™ (Arch Chemicals, Inc), Hydra'Flow™ (Sochibo), Hydrom moist L™ (Symrise), RenovHyal™ (Soliance), Seamoss™ (Biotech Marine), Argireline™ (trade name of the acetyl hexapeptide-3 of Lipotec), spilanthol or an extract of *Acmella oleracea* known under the name Gatuline Expression™, an extract of *Boswellia serrata* known under the name Boswellin™, Deepaline PVB™ (Seppic), Syn-AKE™ (Pentapharm), Ameliox™, Bioxilift™ (Silab), PhytoCellTec™ Argan (Mibelle), Papilactyl D™ (Silab), Preventhelia™

(Lipotec), Subliskin™ (Sederma), Venuceane™ (Sederma), Moist 24™ (Sederma), Vegesome Moist 24™ (Sederma), Essenskin™ (Sederma), Juvinity™ (Sederma), Revidrate™ (Sederma), Resistem™ (Sederma), Chronodyn™ (Sederma), Kombuchka™ (Sederma), Chromocare™ (Sederma), Calmosensine™ (Sederma), Glycokin factor S™ (Sederma), Biobustyl™ (Sederma), Idealift™
 5 (Sederma), Ceramide 2™, Ceramide A2™ and Ceramide HO3™ (Sederma), Legance™ (Sederma), Intenslim™ (Sederma), Prodizia™ (Sederma), Beautifeye™ (Sederma), or mixtures thereof.

Among the plant extracts which can be combined with the peptide of the invention, there may more particularly be mentioned extracts of Ivy, in particular English Ivy (*Hedera Helix*), of *Bupleurum chinensis*, of *Bupleurum Falcatum*, of arnica (*Arnica Montana L*), of rosemary (*Rosmarinus officinalis N*), of marigold (*Calendula officinalis*), of sage (*Salvia officinalis L*), of ginseng (*Panax ginseng*), of ginko biloba, of St.-John's-Wort (*Hypericum Perforatum*), of butcher's-broom (*Ruscus aculeatus L*), of European meadowsweet (*Filipendula ulmaria L*), of big- flowered Jarva tea (*Orthosiphon Stamincus Benth*), of algae (*Fucus Vesiculosus*), of birch (*Betula alba*), of green tea, of cola nuts (*Cola Nipida*), of horse-chestnut, of bamboo, of *Centella asiatica*, of heather, of fucus, of willow, of mouse-
 15 ear, of escine, of cangzhu, of *chrysanthellum indicum*, of the plants of the *Armeniacea* genus, *Atractylodis Platicodon*, *Sinnomenum*, *Pharbitidis*, *Flemingia*, of *Coleus* such as *C. Forskohlii*, *C. blumei*, *C. esquirolii*, *C. scutellaroides*, *C. xanthantus* and *C. Barbatus*, such as the extract of root of *Coleus barbatus*, extracts of *Ballote*, of *Guioa*, of *Davallia*, of *Terminalia*, of *Barringtonia*, of *Trema*, of *antirobia*, *cecropia*, *argania*, *dioscoreae* such as *Dioscorea opposita* or Mexican, extracts of *Ammi visnaga*, of *Siegesbeckia*, in particular *Siegesbeckia orientalis*, vegetable extracts of the family of *Ericaceae*, in particular bilberry extracts (*Vaccinium angustifolium*) or *Arctostaphylos uva ursi*, *aloe vera*, plant containing sterols (e.g., phytosterol), Manjistha (extracted from plants of the genus *Rubia*, particularly *Rubia Cordifolia*), and Guggal (extracted from plants of the genus *Commiphora*, particularly *Commiphora Mukul*), kola extract, chamomile, red clover extract, Piper methysticum
 25 extract (Kava Kava™ from SEDERMA), *Bacopa monieri* extract (Bacocalmine™ from SEDERMA) and sea whip extract, extracts of *Glycyrrhiza glabra*, of mulberry, of *melaleuca* (tea tree), of *Larrea divaricata*, of *Rabdosia rubescens*, of *Euglena gracilis*, of *Fibraurea recisa Hirudinea*, of Chaparral *Sorghum*, of sun flower extract, of *Enantia chlorantha*, of Mitracarpe of *Spermacoceae* genus, of *Buchu barosma*, of *Lawsonia inermis L.*, of *Adiantum Capillus-Veneris L.*, of *Chelidonium majus*, of *Luffa cylindrica*, of Japanese Mandarin (*Citrus reticulata Blanco var. unshiu*), of *Camelia sinensis*, of *Imperata cylindrica*, of *Glaucium Flavum*, of *Cupressus Sempervirens*, of *Polygonatum multiflorum*, of loveyly *hemsleya*, of *Sambucus Nigra*, of *Phaseolus lunatus*, of *Centaureum*, of *Macrocyctis Pyrifera*, of *Turnera Diffusa*, of *Anemarrhena asphodeloides*, of *Portulaca pilosa*, of *Humulus lupulus*, of *Coffea Arabica*, of *Ilex Paraguariensis*, of *Globularia Cordifolia*, of *Albizzia julibrissin*,
 35 *Oxydendron arboretum* or of *Zingimber Zerumbet Smith*.

The compositions of the present invention may include other peptides, including, without limitation, the di-, tri-, tetra-, penta-and hexapeptides and their derivatives. According to a particular

embodiment, the concentration of the additional peptide, in the composition, ranges from $1 \times 10^{-7}\%$ and 20%, preferably from $1 \times 10^{-6}\%$ and 10%, preferably between $1 \times 10^{-5}\%$ and 5%, by weight.

According to the present invention, the term "peptide" refers to peptides containing 10 amino acids or less, their derivatives, isomers and complexes with other species such as a metal ion (e.g. copper, zinc, manganese, magnesium, and others). The term "peptides" refers to both natural peptides and synthetic peptides. It also refers to compositions that contain peptides which are found in nature, and/or are commercially available.

Suitable dipeptides for use within the scope of the present invention include but are not limited to carnosine (beta-AH), YR, VW, NF, DF, KT, KC, CK, KP, KK or TT. Non limitative suitable tripeptides for use herein include, but are not limited to RKR, HGG, GHK, GKH, GGH, GHG, KFK, KPK, KMOK, KMO₂K or KAvaK. Non limitative suitable tetrapeptides for use herein include but are not limited to RSRK (SEQ ID NO: 1), GQPR (SEQ ID NO: 2) or KTFK (SEQ ID NO: 3). Non limitative suitable pentapeptides include, but are not limited to KTTKS (SEQ ID NO: 4) and hexapeptides include but are not limited to GKTTKS (SEQ ID NO: 5) and VGVAPG (SEQ ID NO: 6).

Other suitable peptides for use in the context of the present invention include, but are not limited to: lipophilic derivatives of peptides, preferably palmitoyl derivatives, and metal complexes as aforementioned (e.g. copper complex of the tripeptide HGG). Preferred dipeptide derivatives include N-Palmitoyl-beta-Ala-His, N-Acetyl-Tyr-Arg-hexadecylester (Calmosensine™, Idealift™ from Sederma). Preferred tripeptide derivatives include in particular the N-Palmitoyl-Gly-Lys-His, and Pal-Gly-His-Ly, (Pal-GKH and Pal-GHK from Sederma), the copper derivative of HGG (Lamin™ from Sigma), Lipospondin (N-Elaidoil-KFK) and its analogues of conservative substitution, N-Acetyl-RKR-NH₂ (Peptide CK+), N-Biot-GHK (from Sederma), Pal-KMO₂K (Sederma) and derivatives thereof. Suitable tetrapeptide derivatives for use according to the present invention include, but are not limited to, N-palmitoyl-GQPR (SEQ ID NO: 7) (from Sederma), Ela-KTFK (SEQ ID NO: 8). Suitable pentapeptide derivatives for use herein include, but are not limited to, N-Palmitoyl-KTTKS (SEQ ID NO: 9) (available as Matrixyl™ from Sederma), N-Palmitoyl-Tyr-Gly-Gly-Phe-X (SEQ ID NO: 10) with X Met or Leu or mixtures thereof. Suitable hexapeptide derivatives for use herein include, but are not limited to, N-Palmitoyl-VGVAPG (SEQ ID NO: 11), Pal-GKTTKS (SEQ ID NO: 12) and derivatives thereof. The mixture of Pal-GHK and Pal-GQPR (SEQ ID NO: 7) (Matrixyl™ 3000, Sederma) can also be mentioned.

The preferred compositions commercially available containing a tripeptide or a derivative include Biopeptide-CL™, Maxilip™, Biobustyl™, Procapil™ and Matrixyl™synthe'6™ of Sederma. The compositions commercially available preferred sources of tetrapeptides include Rigin™, Eyeliss™, Matrixyl™ Reloaded and Matrixyl 3000™ which contain between 50 and 500 ppm of Palmitoyl-GQPR (SEQ ID NO: 7) and carrier, proposed by Sederma.

The following marketed peptides can be mentioned as well as additional active ingredients: Vialox™, Syn-ake™ or Syn-Coll™ (Pentapharm), Hydroxyprolisilane CN™ (Exsymol), Argireline™, Leuphasyl™, Aldenine™, Trylgen™, Eyeseryl™, Serilesine™ or Decorinyl™ (Lipotec), Collaxyl™ or Quintescine™ (Vincienc), BONT-L-Peptide™ (Infinitec Activos), Cytokinol™LS (Laboratoires Serobiologiques/Cognis), Kollaren™, IP2000™ or Meliprene™ (Institut Européen de Biologie Cellulaire), Neutrazen™ (Innovations), ECM-Protect™ (Atrium Innovations), Timp-Peptide™ or ECM Moduline™ (Infinitec Activos).

The present invention also provides a topical cosmetic or dermatopharmaceutical treatment method to improve the appearance and condition of the skin and its appendages, comprising the topical application to the skin of a subject in need thereof of an effective amount of meristematic cells or of their cellular extract according to the invention or of a composition according to the invention comprising said cells or said cellular extracts as recited above.

"Topical treatment" or "topical use" means an application that is intended to act where it is applied: skin, mucous, appendages.

The meristematic cells, the cellular extract or composition according to the invention can be applied locally applied to targeted areas.

The "effective" amount depends on various factors, such as the age, the condition of the patient, the severity of the disorder or disease and the administration mode. An effective amount means a non-toxic amount enough to achieve the desired effect.

In a cosmetic composition according to the invention, the meristematic cells or cellular extract to be present in an effective amount, are generally present in an amount ranging from 0.000001% and 15% based on the total weight of the composition, preferably between 0.0001% and 10% depending on the destination of the composition and the more or less pronounced desired effect.

According to preferred features of the invention:

- The physiologically acceptable medium is a hydrophilic matrix wherein said cells are in suspension; and/or
- The composition comprises a thickening agent and/or undergoes high pressure homogenization; and/or
- The composition, forming a cosmetic ingredient, includes at least 0.1% of phenylpropanoid glycosides, usually approximately 0.1%. This ingredient is then used for example in an amount of several % to 20% to prepare cosmetic formulations.

All percentages and ratios used herein are by weight of the total composition and all measurements are made at 25°C unless it is otherwise specified.

For example, for a face cosmetic treatment, the SCCS'S (Scientific Committee on Consumer Safety) Notes of Guidance for the testing of cosmetic substances and their safety evaluation (8th Revision, 11 dec. 2012) has set a standard amount for applying a cream of 2.72 mg/cm²/day/person and for a body lotion of 0.5 mg/cm²/day/person.

According to other specific features, the cosmetic treatment method according to the invention can be combined with one or more other treatment methods targeting the skin such as lumino-therapy, heat or aromatherapy treatments.

According to the invention, devices with several compartments or kits may be proposed to apply the method described above which may include for example and non-restrictively, a first compartment containing a composition comprising the active cells of the invention, and in a second compartment an excipient and/or an additional active, the compositions contained in the said first and second compartments in this case being considered to be a combination composition for simultaneous, separate or stepwise use in time, particularly in one of the treatment methods recited above.

- 10 The method of treatment according to the invention is particularly suitable for:
- An anti-aging treatment via an action on the dermal and epidermal MEC molecules; and/or
 - A dermal densification treatment to densify the dermis via the stimulation of collagen I and elastin; and/or
 - Slowing down the degradation of the molecules of the dermal extracellular matrix; and/or
 - 15 - Acting on the DEJ by stimulating collagen IV and/or laminin; and/or
 - Thickening the epidermis; and/or
 - The treatment of age spots; and/or
 - An anti-wrinkle treatment; and/or
 - A volumizing treatment.
- 20 Other cosmetic applications are also possible, for example, for a treatment for slimming, loss of elasticity, detoxification, anti-glycation, tensing, anti-fatigue, for a treatment of under-eyes bags and/or dark circles, soothing, firming, treatment of hair and body hairs, action on skin radiance etc. for a preventive or curative action.

DETAILED DESCRIPTION

- 25 The present invention will be better understood and other advantages will become apparent from the following detailed description made with reference to the annexed drawings, on which Figure 1 shows a skin deformation graph using a CutometerTM illustrating the different measured parameters.

IRB PL3 cell line creation

- 30 Selected pieces of leaves of *Plantago lanceolata* genus are taken, washed and cut into small pieces of several cm, in order to achieve 2000 to 5000 explants. After a series of decontamination treatment and then of sterilization, the pieces are placed on an agar culture medium in the presence of a nutrient medium containing plant growth hormones to induce callogenesis (callus formation).

- After a suitable period of time, a dedifferentiated cell mass or callus is formed and transferred on a larger area and in a fresh culture medium to be able to multiply. A number of subcultures (transfers on a fresh culture medium) is performed for stabilizing the cell line, i.e. until it has a high and constant speed of proliferation, preservation of phenotype and a constant content of bioactive compounds of interest (primary and secondary metabolites).
- 35

The cell line is subsequently subjected to a selection step of cultivating the cells for an appropriate period, collecting the formed cell aggregates and inoculating these on a liquid culture medium for a period enough to obtain the multiplication of cellular aggregate. The best cell line will be the one giving as quickly as possible and in a reproducible manner a large biomass having an optimal content in selected metabolites, the best biological activity and a homogeneous phenotype.

The inventors have selected the IRB PL3 *Plantago lanceolata* cell line.

This cell line was also selected for its ability to produce phenylpropanoid glycosides in an amount of about 10% as measured by weight of total phenylpropanoid glycosides expressed as plantamajoside relative to the dry cell weight.

10 **A) Industrial process for obtaining a biomass of *Plantago lanceolata* meristematic cells obtained from the IRB PL3 *Plantago lanceolata* cell line or an extract of said cells**

The *Plantago lanceolata* IRB PL3 line is initially multiplied to obtain sufficient biomass of meristematic cells to perform the step of large-scale production.

The following steps are implemented:

- 15 a) Inoculation of the selected line in liquid medium and culture for a sufficient time to increase the biomass of at least 300%;
- b) Optionally, transfer the suspension obtained in a) in a fresh liquid medium and again culture for a sufficient time to obtain a biomass increase of at least 300% time;
- c) optionally repeating step b)
- 20 d) Transfer of cell suspensions obtained at steps a) to c) in a bioreactor with fresh liquid medium, and conduct the culture under conditions such as and for a time sufficient to obtain a cell biomass containing the metabolite of interest, i.e. the PPG in sufficient amount, this step of bioreactor production comprising an step elicitation achieved by modifying the content of nutrients of the culture medium.

25 **The bioreactor:**

Volume: 5 to 50 times larger than the volume of biomass used as inoculum; smooth and uniform internal surface bioreactor (no edges or angles which could cause the rupture of cell walls).

Culture conditions:

30 Culture medium: medium comprising mineral salts (macronutrients and micronutrients solution), vitamins, plant hormones and sucrose. Vegetable agar is added to solid media.

Temperature: between 15°C and 35°C, preferably between 20°C and 30°C, and more preferably at 25°C.

Duration: between 7 and 21 days, preferably between 10 and 14 days.

35 Biomass agitation: it is important that biomass be optimally ventilated and at the same time be kept stirred either by internal means or by an external means. It is necessary that the agitation, although small, be effective, especially in the final steps, when the biomass is in large quantities. For purposes of the present invention, suitable agitation by internally means are propellers rotating between 20 and 120 rpm,

preferably 60 rpm, or externally by orbital agitation means rotating preferably between 40 and 200 rpm and preferably about 120 rpm.

Oxygenation: usually carried out using sterile air, at a rate of 0.5 to 4 liters per minute, preferably between 2 and 2.5 liters per minute, for a volume of 10 liters of biomass. Alternatively, gas mixtures containing from 10% to 100% v/v of oxygen may be used. It is preferable to use diffusion means of air or oxygen with a nozzle having a flow rate of between 10ml/min and 600ml/min and preferably between 50ml/min and 350ml/min.

Treatment of the obtained cellular medium

Filtration, to remove the culture medium and recover the cell biomass. This biomass may be characterized by an equivalent rate of lyophilized cells.

Characterization of the active compounds contained in the cells by analytical determination of primary and secondary metabolites produced by the culture, including protein and PPG.

In option:

- Drying of the cells in particular by lyophilizing or atomization to provide greater stability of the compounds of interest, improve the long-term storage without the need to add preservatives.
- High pressure homogenization of the cellular culture medium: reduces the size of cellular aggregates, certain cells can be broken and a mixture of whole cells and crushed cells can be obtained;
- Extraction of the content of the cells by crushing/lysis/disruption of the cells in a suitable solvent and separating the liquid and solid phases (by centrifugation or filtration or the like) to obtain a cellular extract.

B) Preparation of a composition according to the invention

The cell biomass, either as obtained at point B) after filtration, or in a dried form, or the cellular extract, can be mixed in a physiologically acceptable medium forming the excipient.

As a preferred example, the physiologically acceptable medium is a hydrophilic matrix in which the cells are suspended, for example in the case of a cosmetic composition glycerol and/or butylene glycol. Additives may also be added if necessary, such as antimicrobial agents, antioxidants, stabilizing agents, agents acting on pH, emulsifying agents or thickeners, such as a xanthan gum thickener which will promote the maintaining of the cells in suspension.

A supplementary high-pressure homogenization step can be provided to obtain a fine particle suspension.

An ingredient for cosmetic purposes, concentrated in active compounds can thus be formed comprising for example 20% by weight of fresh biomass of whole meristematic cells (corresponding to about 1% of dry cells) relative to the total weight of the composition, having a final content of about 0.1% of phenylpropanoid glycosides, in a physiologically acceptable excipient mixture consisting of glycerol (approximately 80%) and xanthan gum (0.3 wt%).

This ingredient can then be used in an amount of several % to 20% to prepare cosmetic formulations as set out below in Galenic F).

C) In vitro evaluations

In vitro evaluations were performed from an ethanol extract concentrated 17 times compared to the ingredient according to the invention defined above at point C) with 1% of lyophilized cells (i.e. 17% of freeze-dried cells). This extract is titrated at 1.45% of total phenylpropanoid glycosides. This extract (called “product of the invention” in the *in vitro* evaluations) was tested thereafter at 0.06%, corresponding to a concentration of phenylpropanoid glycosides (PPG) of 8.8 ppm. When this % is different, it is specified.

1) Dermal densification

a) **Collagen I synthesis on human dermal fibroblasts (NHF)**

Collagen I is the most abundant protein in the dermis. It is an essential component to have a firm and elastic skin and tends to be less produced with increasing age.

NHF in culture were exposed to the invention product for 6 days. Collagen I synthesis was measured using photography after immune-labelling. Cellular nuclei were counted using a DNA stain (Hoescht 33258) to standardize the data.

Table 1: Variation in collagen I synthesis by HDF (n=3).

	Collagen I (AFU*/10 ⁶ cell.)	Variation (%)
Negative control	41.4 ± 14.5	Reference
Invention product	200.6 ± 20.1	+385%; <i>p</i><0.01

* AFU: arbitrary fluorescence unit; no toxicity was noted compared to the control.

b) **Elastin synthesis**

Elastin is synthesized and secreted in the extracellular space by dermal fibroblasts first in pro-elastin, then in tropo-elastin. Elastin is the major component up to 90% of elastic fibers. Elastin and fibrillin forming the elastic fibers, and collagen, are the main constituents of the extracellular matrix. It is important to stimulate their synthesis which decrease with age.

Normal human fibroblasts (NHF) were grown in contact or not with the test products at various concentrations. Elastin synthesis produced by the cells was then quantified by immuno-labeling and image analysis on the fixed layers. A nuclear counter staining with Hoechst reagent complements the study and can be used to weight the results.

Table 2: Variation of elastin synthesis by NHF (n=3).

	Elastin (AFU*/10 ⁶ cell.)	Variation (%)
Negative control	3.22 +/- 0.89	Reference
Invention product	8.46 +/- 3.61	+163%; <i>p</i><0.01

* AFU: arbitrary fluorescence unit; no toxicity was noted compared to the control.

c) **Elastin synthesis on skin explants after accelerated ageing**

Woman skin explants (53-year-old) underwent accelerated ageing. Elastin having slow renewal, it is difficult to visualize the neo-synthesis without this operation. To do this, the explants temporarily

received a corticosteroid, and then a cream according to the invention (Galenic example recited at point E)) or a placebo cream was applied for 9 days. Skin sections were then analyzed under a microscope after immune-labelling of Elastin. 30 photos (n= 10/3 photos per case) were used to quantify and compare the respective quantities of elastin.

5 **Table 3:** Variation of elastin synthesis on treated skin explants (n=3)

Placebo not stressed	« Aged » placebo Corticosteroid stress	« Aged » invention product Corticosteroid stress
Reference	-25%; $p < 0.01$	+76%; $p < 0.01$

The application of the corticosteroid reduces the elastin level by 25% with the placebo. The invention product allows raising it up to a higher level than the level of unstressed placebo skins.

d) Hyaluronic acid synthesis on human fibroblasts

10 Hyaluronic acid is an essential component of the dermis. The interest of hyaluronic acid lies in its viscoelastic properties and its ability to capture water. Thus water fills the spaces between collagen and elastin fibers in the dermis. This contributes to skin flexibility and prevents wrinkle formation. This substance decreasing with age, skin becomes dry and wrinkles.

15 Normal human fibroblasts (NHF) were contacted or not with the invention product. Hyaluronic acid synthesis produced by the cells was then quantified by immunofluorescence and image analysis on the fixed layers. A nuclear counter staining with Hoechst reagent complements the study and can be used to weight the results.

Table 4: Variation of hyaluronic acid synthesis by NHF (n=3)

hyaluronic acid IMF NHF	Variation cell Nb (HXT)	Hyaluronic acid (AFU/10 ⁶ cell)	Variation (%)
Control	Reference	17.07 +/- 12.40	Reference
Invention product	-3	52.92 +/- 15.90	+210%; $p < 0.01$

The product according to the invention stimulates the hyaluronic acid synthesis by normal human fibroblasts.

20 e) Collagens, elastin and miRNAs

NHF (human dermal fibroblasts) were contacted with the product of the invention for 3 and 24 hours. At each time point the miRNAs were extracted and were studied by transcriptional analysis after checking the quality of RNA by capillary electrophoresis and determining the miRNA percentage. The values obtained at each time point were compared with the control without product.

25 **Table 5:** Variation in miR-29 and miR-196a ratios on NHFs

miRNA type	Negative control (AFU*)	Variation* at 3h	Variation* at 24h
miRNA-29	4886	0.766	0.647
miRNA-196a	168	0.746	0.660

* AFU: arbitrary fluorescence unit; AFU variation considered case/control AFU; no toxicity was observed compared to the control.

This study shows that the product according to the invention reduces the expression of known miRNA-29 levels to slow down the synthesis of collagens and elastin as well as miRNA-196a which suppress the collagen I synthesis.

These observations were reinforced by the decrease of the expression of miRNA-25 and -150 also repressors of these syntheses. These results explain why the product according to the invention promotes the synthesis of dermal macromolecules: collagen I and IV, elastin, and thus promotes dermal densification.

f) Slowing down the degradation of extracellular matrix molecules

Dermal proteases are induced by various stresses and during ageing. They contribute to fragmentation and increased degradation of dermal macromolecules (collagen I in particular), rendering skin less firm, less dense and less flexible. MMPs are controlled by different mechanisms including TIMPs produced by the cells. These TIMPs are glycoproteins responsible for inhibiting the activity of MMPs by complexation. They are also controlled by miRNAs.

On NHFs having undergone oxidative stress (H_2O_2) mimicking accelerated ageing (SIPS = Stress Induced Premature Senescence) were measured on the one hand the production of MMP-1 and -2 (by ELISA) and on the other hand the production of TIMP-1 and -2 (by ELISA) in the presence or absence of the product according to the invention.

Oxidative stress causes on the control an increase in MMP-1 and -2 (+21%; +21%) and a decrease in TIMP-1 and -2 (-30% and -41%).

Table 6: Variation of the MMP-1 and -2 contents, and of the TIMP-1 and -2 contents produced by NHFs (n=4* or 3**) following an oxidative stress

	Control	Invention product	Variation (%)
MMP-1 (ng/10⁶ cell.)*	5049 ± 862	3426 ± 318	-32%; $p < 0.05$
MMP-2 (ng/10⁶ cell.)*	952 ± 81	734 ± 61	-23%; $p < 0.01$
TIMP-1 (ng/10⁶ cell.)**	8665 ± 664	11006 ± 1870	+27%; $p < 0.05$
TIMP-2 (ng/10⁶ cell.)**	129 ± 12	156 ± 10	+21%; $p < 0.05$

No toxicity was observed compared to the control

These results show that the production of MMP-1 and -2 can be controlled by the invention product, by respectively reducing production of -32% and -23% ($p < 0.05$ or 0.01), and in parallel production of natural inhibitor TIMP-1 and -2 can be stimulated by the invention product of +27 and +21% ($p < 0.05$).

g) MMP, TIMP and miRNA

MicroRNA-21 is known to repress TIMP-1 production and to facilitate MMPs action.

NHFs were contacted with the invention product for 3 and 24 hours. Each time the miRNAs were extracted and quantified as mentioned above.

Table 7: Variation miR-21 ratios in NHFs

miRNA type	Negative control (AFU*)	Variation* at 3h	Variation* at 24h
miR-21	3609	0.757	0.657

* AFU: arbitrary fluorescence unit; AFU variation considered case/control AFU; no toxicity was observed compared to the control.

The decrease of miR-21 expression will increase TIMP-1 production and thus will reduce MMPs activity. The product according to the invention thus advantageously protects the dermal macromolecules against degradation by proteases.

Therefore, in parallel with its stimulatory effect on the synthesis of matrix macromolecules seen above, the product according to the invention protects the dermal extracellular matrix from degradation by proteases. This is particularly sought for anti-aging action.

2) Maintaining JDE integrity and thus the anchorage of human keratinocytes (and melanocyte control).

DEJ or dermo-epidermal junction is a thick membrane that ensures the cohesion between the dermis and the epidermis.

a) **Collagen IV synthesis stimulation on skin explants**

Collagen IV is a type collagen mainly located at the basal membrane or dermo-epidermal junction (DEJ). It is one of the essential elements of the skin, not by quantity but by its role at the level of the DEJ. With age, collagen IV is more fragmented because attacked by MMPs and at the same time less synthesized, like laminins, resulting in some areas in an impaired JDE, disturbed relationships between melanocytes, keratinocytes and JDE, resulting in increased skin pigmentation.

Abdominal skin explants from a 53-year-old woman were placed in survival in an appropriate medium and received a cream according to the invention (galenic formula n°1 of point F)) or a placebo cream every day for 9 days. At the end of this contact period, sections of the skin were taken and collagen IV has been labelled with a fluorescent antibody. 30 photos (n=10/3 photos per case) were used to quantify and compare the respective quantities of collagen IV.

Table 8: Variation of collagen IV production on skin explants (n=3)

Collagen IV Explants	Mean fluorescence intensity*	% Variation / placebo
Placebo	11.0 +/- 2.5	Reference
Invention product	16.8 +/- 2.9	+53.2%; $p < 0.01$

* AFU: arbitrary fluorescence unit

The product according to the invention therefore advantageously stimulates the synthesis of collagen IV on skin explants.

b) Laminin synthesis by human keratinocytes

Basal keratinocytes are attached to each other by forming a thin layer bonded to the dermis *via* the DEJ and its complex network of proteins and fibers. Among them are the laminins, boat anchor-shaped proteins, whose branches bind together collagen IV, dermal proteoglycans and keratinocytes.

- 5 Laminins decrease in quantity with age and at the level of senile lentigo. Thus stimulating their synthesis is of great interest.

Human keratinocytes were contacted with the invention product during 3 days. A dosage of laminin was carried out on culture supernatants using an ELISA method. An estimate of cell amount by Hoechst assay was used to weight this dosage.

- 10 Table 9: Variation of laminin synthesis (n=5).

	Laminins (ng/10 ⁶ cell.)	Variation (%)
Negative control	246 ± 23	Reference
Invention product	360 ± 13	+46%; <i>p</i> <0.01

No toxicity was observed compared to the control

- The results show that laminins are more produced (+46%, *p*<0.01) in the presence of the product according to the invention than in the control. The results complement those obtained with collagen IV. Together, these two essential components of the JDE ensure to basal keratinocytes a better anchoring and help maintaining epidermis flexibility and the control of melanocytes activities.

3) Boosting epidermal metabolisms to thicken the epidermis

Epidermis thinning is one aspect of aging, hence the need to act also in the direction of the reinforcement of epidermis and therefore also to act on the reinforcement of keratinocyte differentiation.

- 20 a) **Marking of neutral lipids with red oil**

In normal epidermis, a mixture of neutral and polar lipids is dominant in the deep layers and is gradually replaced by a more apolar lipidic content, including ceramides, free sterols and free fatty acids, as well as variable quantities of triglycerides, sterol esters and other non-polar components. These neutral lipids (especially ceramides) will form an intercellular cement in the *stratum corneum*, providing its water impermeability.

The rise of neutral lipids is thus well correlated with the final phase of keratinocyte differentiation (corneocyte formation).

- Confluent human keratinocytes were contacted with the product of the invention at 0.12%, in a medium containing calcium, and differentiation was monitored. The neutral lipid synthesis is then evaluated by staining with red oil. The amount of cells was measured by the Hoechst method.

Table 10: keratinocyte labelling with red oil (n=3 and 15 photos per case)

<i>Red oil labelling</i>	Cell nbr. Variation %	Lipids (AU / 10⁶ cells)*	<i>Variation %</i>
Control	Reference	0.75 +/- 0.30	Reference
Invention product	-34%	1.22 +/- 0.31	+63%; $p<0.01$

*Arbitrary unit

The decrease in cell number is classic after contact with a pro-differentiator product and does not reflect cytotoxicity of this product, but rather a lower adhesion to the support of the differentiated cells.

In parallel to its pro-differentiator effect (observable on cell layers), the product of the invention stimulates the synthesis of epidermal lipids. This result is consistent because differentiation is accompanied by accumulation of ceramides which are neutral lipids.

b) Reinforcement of tight junctions: ZO-1 (TJP-1) and claudin-1

Tight junctions form a protection system which strongly binds the cells together via the actin network in the upper part of the epidermis. They have a role as guardian of the water homeostasis, preventing water evaporation. ZO (zona occludens) -1 and claudin-1 are involved in this network.

Human keratinocytes were contacted with the invention product at 0.12% for 2 days and immunofluorescent labelling of ZO-1 and claudin-1 was carried out. A nuclear counter staining with Hoechst dye associated with a count is used to weight the data.

Tables 11 and 12: Variation in claudin-1 and ZO-1 amounts on keratinocytes (n=3 and 18 photos per case)

<i>Claudin-1 IMF HPEK</i>	<i>Cell nb. variation (HXT)</i>	<i>Claudin-1 (AFU/500cell)*</i>	<i>Variation %</i>
Control	Reference	18459 +/- 8354	Reference
Invention product	-1	41611 +/- 13459	+125%; $p<0.01$

<i>ZO-1 (TJP-1) IMF HPEK</i>	<i>Cell nb. variation (HXT)</i>	<i>ZO-1 (AFU/500cell)*</i>	<i>Variation %</i>
Control	Reference	36085 +/- 8653	Reference
Invention product	+2	51227 +/- 13210	+42%; $p<0.01$

*AFU: arbitrary fluorescence unit

The compound of the invention induces a significant increase of two proteins of tight junctions: ZO-1 and claudin-1 at the level of the corneocytes and therefore contributes to strengthen the skin barrier and the cohesion of the epidermal cells.

c) Loricrin and filaggrin synthesis

Loricrin and filaggrin are two specific proteins of keratinocyte differentiation. The effect of the product according to the invention on their synthesis was evaluated.

Confluent human keratinocytes were contacted with the product of the invention at 0.12% in a medium containing calcium, and differentiation was followed. After culture, the layers are rinsed, fixed and loricrin and filaggrin synthesis are identified by immunofluorescence and quantified by image analysis. An Hoechst counter staining of nuclei completes the study and weights these results.

- 5 Table 13: Effect of the invention product on loricrin synthesis on human keratinocytes (n=3 and 15 photos per case)

<i>Loricrin IMF</i>	<i>Cell nb. variation (HXT)</i>	<i>Loricrin (AFU/10⁶ cell)</i>	<i>Variation %</i>
Control	Reference	0.20 +/- 0.29	Reference
Invention product	-37	1.54 +/- 1.57	+670%; $p<0.01$

Table 14: Effect of the invention product on filaggrin synthesis on human keratinocytes (n=3 and 15 photos per case)

<i>Filaggrin IMF</i>	<i>Cell nb. variation (HXT)</i>	<i>Filaggrin (AFU/10⁶ cell)</i>	<i>Variation %</i>
Control	Reference	0.04 +/- 0.04	Reference
Invention product	-37	0.34 +/- 0.24	+674%; $p<0.01$

- 10 The decrease in cell number is a classic phenomenon after contact with a pro-differentiator product and does not reflect cytotoxicity of this product, but rather a lower adhesion to the support of the differentiated cells.

The product according to the invention strongly stimulates the filaggrin and loricrin synthesis, and therefore differentiation of keratinocytes.

d) Hyaluronic acid (HA) differentiation of keratinocytes

- 15 Thinning and drying of the epidermis is due to HA deficiency. This happens with aging. Human keratinocytes are cultured for 24hrs. The cells are contacted or not with the product according to the invention at 0.12% for 3 days. The culture supernatants are removed and a hyaluronic acid assay is performed by ELISA, an estimate of the amount of cells by a BCA assay (measuring the amount of protein) is used to weight the results.

- 20 Table 15: Effect of the invention product on hyaluronic acid synthesis on human keratinocytes (n=5)

<i>HA Keratinocytes</i>	<i>Cell nb. variation (BCA)</i>	<i>Hyaluronic acid (ng/10⁶ cell)</i>	<i>Variation %</i>
Control	Reference	4728.7 +/- 537	Reference
Invention product	-5	6373.1 +/- 689.6	+35%; $p<0.01$

The product according to the invention significantly stimulates the synthesis of HA in keratinocytes.

e) **Stimulation of CD44 synthesis**

CD44 is a surface receptor of HA involved in interactions between cells and cell adhesion. The decrease of HA and CD44, during aging, is accompanied by a decrease in keratinocyte proliferation. Keratinocytes are cultured. After a growth phase of one week, the confluent cells are contacted with the product according to the invention at 0.12% during 48h. After the contact, the layers are rinsed, fixed and CD44 revealed by immunofluorescence. The labelled layers are then photographed and the specific labelling of each protein of interest is quantified by image analysis using Image J™ software. A counter staining of nuclei with Hoechst dye associated with a count is used to weight these data.

Table 16: Effect of the product of the invention on CD44 amount on human keratinocytes (n=3 and 18 photos per case)

<i>CD44 IMF keratinocytes</i>	<i>Cell nb. variation (HXT)</i>	<i>CD44 (AFU/500cell)</i>	<i>Variation %</i>
Control	Reference	10788 +/- 9059	Reference
Invention product	-5	61146 +/- 16000	+467%; $p<0.01$

The product according to the invention increases the synthesis of CD44. This effect combined with the effect of stimulating the synthesis of hyaluronic acid participates in improving the hydration of the epidermis, and increase its thickness.

4) **Decrease of pre-inflammatory messengers, known to accelerate ageing**

The cells produce pre-inflammatory mediators more significantly with age. IL-6 for example, is a cytokine whose serum levels are increased in elderly; IL-6 also induces premature senescence in human fibroblasts. On their side, PGE2 form a critical compound for senescence induction and for maintaining an inflammatory condition; they also occur on melanocytes by increasing tyrosinase activity and dendricity which produces hyperpigmentation. Also aged skin cells produce more IL-6 and IL-8 in response to a stress than young cells. Thus, a decrease in production of these inflammatory mediators in the basal state in the absence of stress, is advantageous in an anti-ageing perspective.

Normal human keratinocytes are cultured until a confluent. At this stage, they are contacted with the products to be tested for 48 hours. The quantities of synthesized PGE2, IL-6 and IL-8 were measured in the culture supernatants by ELISA. The number of cells was assessed by a test of metabolic vitality of MTT reduction (delta Do at 570nm).

Table 17: Variation of PGE₂, IL-6 and IL-8 amounts produced by keratinocytes (n=3).

	PGE₂ (pg/10⁶ cell.)	Variation (%)
Control	266 ± 50	Reference
Invention product: 0.15%	148 ± 4	-44%; $p<0.01$

	IL-6 (pg/10⁶ cell.)	Variation (%)
Control	111 ± 10	Reference
Invention product: 0.15 %	10 ± 0.5	-91 %; $p < 0.01$
	IL-8 (pg/10⁶ cell.)	Variation (%)
Control	1588 ± 250	Référence
Invention product: 0.15 %	561 ± 42	-65 %; $p < 0.01$

No toxicity was observed compared to the control

Thus, advantageously the product according to the invention reduces strongly and significantly pre-inflammatory messengers that promote ageing.

5) Generic anti-senescence signals

a) **Antioxidant axis**

DPPH (1,1-diphenyl-2-picryl hydrazyl) is a stable free radical which is widely used for the detection of free radical scavengers. This molecule while losing its radical character is converted into DPPH2 (1,1-diphenyl-2-picryl hydrazine). This conversion is accompanied by discoloration (violet to yellow), which can be followed in time by spectrophotometry at 517nm. Control having no scavenging activity maintains a constant optical density.

Cell membranes are composed of oxidizable phospholipids. Using a cell-free model (unsaturated phospholipid based liposomes) and a reproducible and physiological oxidative stress (UVA radiation), it is possible to track by spectrophotometry (at 233nm) the formation of the lipoperoxidation early by-products that are diene conjugates. An anti-lipoperoxidant agent can reduce or even eliminate this phenomenon.

Table 18: Measure of antioxidant potential of the invention product (n=6)

	DPPH*	Variation (%)	Lipoperoxydation*	Variation (%)
Negative control	0.488 ± 0.004	Reference	2.006 ± 0.015	Reference
Invention product	0.337 ± 0.006	-31 %; $p < 0.01$	0.712 ± 0.040	-65 %; $p < 0.01$

*AU: arbitrary unit

The product according to the invention advantageously has a good effect against free radicals and inhibits lipid peroxidation induced by UVA.

b) **Action against oxygen reactive species (ROS)**

ROS excess in the cell leads to the damage increase and at long-term to cellular aging.

ROS evaluation was performed on human dermal fibroblasts (NHF) with the DCFH-DA probe that, once in the cell, becomes fluorescent in contact with ROS (fluorescence level directly proportional to the ROS amount). The fluorescence obtained in the cells in contact with the invention product was quantified and compared with that of the control without product. Raw results were reduced to the number of cells.

Table 19: Variation of intracellular ROS production (n=3).

	Fluorescence (UFA*/10 ⁴ cell.)	Variation (%)
Negative control	3527 ± 25	Reference
Invention product	804 ± 33	-77%; <i>p</i> <0.01

* AFU: arbitrary fluorescence unit; AFU variation considered case/control AFU; no toxicity was observed compared to the control.

The product according to the invention thus advantageously protects fibroblast against ROS that may damage its lipid, protein or nucleic structures.

c) Induction of superoxide dismutase (SOD)

SOD is a metalloenzyme which is part of the defense system of the cell against free radicals by performing superoxide anion dismutation into oxygen and hydrogen peroxide. Mitochondria contain a manganese superoxide dismutase (SOD2) which represents the first anti-ROS defense line. Mice skin deficient in this enzyme is very impaired and prematurely aged, in particular epidermis is thinner. The amount of SOD2 was assessed by ELISA in the HDF cell layers after contact with the product according to the invention and compared to the control without product. Raw results were reduced to cell number.

Table 19: Variation of intracellular SOD2 amount (n=3).

	SOD2 (U*/10 ⁶ cell.)	Variation (%)
Negative control	21.8 ± 2.1	Reference
Invention product	28.9 ± 0.5	+33%; <i>p</i> <0.01

* Activity unit; no toxicity was observed compared to the control

The product according to the invention increases basally cell SOD activity and therefore advantageously enhances their potential of defense against oxidation.

d) Protection with regard to protein glycation

Protein glycation increases with age; it affects all skin proteins (structural or functional) and in particular dermal proteins such as collagen, disrupting the extracellular matrix and making the skin lose its tone and flexibility. This glycation will generate an increase of radical products which in turn will disrupt the metabolism and degrade cell structures of the skin.

A model protein, the BSA (bovine serum albumin), is placed in the presence of a reducing sugar, in the presence or not of the product according to the invention for one week at 50°C to form AGEs (advanced glycation end products). The glycation end products have a natural fluorescence which is quantified (ex = 360nm and em = 460nm).

The results are given after ultracentrifugation so as to be free of any artefact related to the natural fluorescence of the product.

Table 20: Effect on BSA glycation (n=4).

<i>Glycation</i>	<i>Glycation (AFU)</i>	<i>Variation %</i>
Control	10910 +/- 125	Reference
Invention product	2488 +/- 270	-77%; $p < 0.05$

The product according to the invention decreases the glycation of BSA protein model as from a dose of 0.06%. Thus, in addition to its anti-radical effect, the product of the invention has a protective action with respect to protein glycation.

e) miRNA and senescence

It has been shown that the trio of miR-30e, miR-34a and miR-181a on the one hand, and the miR-152 and miR-181a duo on the other hand, clearly were involved in the increase of senescent phenotypes in neurons or fibroblasts. The decrease of these miRNAs has prolonged cell survival and reduced senescent phenotype appearance. As for the synthesis of collagen, these studies show the importance of the regulation path by miRNAs.

NHF's were contacted with the invention product for 3 and 24 hours. Each time the miRNAs were extracted and were studied by transcriptional analysis after checking the quality of RNA by capillary electrophoresis and determining the percentage of miRNAs. The values obtained at each time point were compared with the control without product.

Table 21: Variation of the miR-30a and -181a quantity in HDF

miRNA type	Negative control (AFU**)	Variation* at 3h	Variation* at 24h
miR-30a	28	0.412	0.457
miR-181a	168	0.752	0.637

** AFU: fluorescence arbitrary unit; * variation considered case AFU/control AFU; no toxicity observed compared to the controls.

Similar results were obtained for the miR-34a and -152.

The product according to the invention reduces expression of miR-30a, 34a, 181a and -152 described in the scientific literature as senescence accelerators. The product of the invention has therefore a profile of anti-aging activities very interesting especially for cosmetics.

D) Comparative results of in vitro studies

The following tests were subjected to a comparison between the product of the invention and pure verbascoside in a concentration equivalent to the concentration of phenylpropanoid glycosides (PPG) of the product according to the invention.

1) On dermal macromolecule synthesis (collagen I and hyaluronic acid)

a) Collagen I synthesis

Normal human fibroblasts (NHF) were cultured for 24 hours. The cells are contacted or not with the test product (product of the invention or verbascoside) for 6 days. Collagen I synthesis produced by the cells is then quantified by immune-labelling and image analysis on the fixed layers. A counter nuclear staining with Hoechst complements the study and is used to weight the results.

Table 22: Compared effect between verbascoside and invention product on collagen synthesis on human fibroblasts

<i>Collagen I IMF</i>	<i>PPG concentration</i>	<i>Cell nb variation</i>	<i>Collagen I (UFA/10⁶ cell)</i>	<i>Variation %</i>
Control	-	Reference	41.36 +/- 14.47	Reference
Invention product	17.6 ppm	+1.8	84.84 +/- 7.38	+105%; $p < 0.01$
Verbascode	17.6 ppm	-16.2	50.32 +/- 15.24	+21.7%; <i>dns</i>

At the studied dose, the product according to the invention strongly stimulates the synthesis of collagen I, whereas verbascoside is inactive.

b) Hyaluronic acid synthesis

Normal human fibroblasts (NHF) were cultured for 24 hours. The cells were contacted or not with the test product (product of the invention or verbascoside). The synthesis of hyaluronic acid produced by the cells was then quantified by immune-labelling and image analysis on the fixed layers. A counter nuclear staining with Hoechst complements the study and was used to weight the results.

Table 23: Compared effect between verbascoside and the invention product on hyaluronic acid synthesis on human fibroblasts

<i>Hyaluronic acid IMF</i>	<i>PPG concentration</i>	<i>Cell nb variation</i>	<i>Hyaluronic acid (AFU/10⁶ cell)</i>	<i>Variation %</i>
Control	-	Reference	17.07 +/- 12.40	Reference
Invention product	8.8 ppm	-3	52.92 +/- 15.90	+210%; $p < 0.01$
Verbascode	8.8 ppm	+2	28.20 +/- 4.85	+65%; $p < 0.05$

At the tested dose, the product according to the invention strongly stimulates the synthesis of hyaluronic acid. Verbascode also has a stimulating effect but much weaker.

In parallel to these tests, it was found, at higher doses of verbascoside, that this molecule had a cytotoxic effect on fibroblasts, whereas at an equivalent concentration in PPG the product according to this invention showed no cytotoxicity. The assessment of the number of cells was made by marking the nuclei with Hoescht dye, and by counting them on a large number of fields.

<i>Cytotoxicity</i>	<i>PPG concentration</i>	<i>Cell nb variation</i>
Control	-	Reference
Invention product	17.6 ppm	-7
	29 ppm	-16
Verbascoside	17.6 ppm	-31
	29 ppm	-44

2) **On the synthesis of molecules that are responsible of the reinforcement of the DEJ and epidermis (laminin and hyaluronic acid)**

Human keratinocytes are cultured for 24hrs. The cells are contacted or not with the test product (product of the invention or verbascoside) for 3 days. Culture supernatants are taken and a dosage of the amount of laminin and hyaluronic acid is conducted by the ELISA method, an estimate of the amount of cells by Hoechst assay (measuring the amount of DNA) or BCA (measure the amount of protein) is used to weight the results.

a) **Laminins**

Table 24: Compared effect between verbascoside and the product of the invention on laminin synthesis on human keratinocytes

<i>Laminin keratinocytes</i>	<i>PPG concentration</i>	<i>Cell nb variation</i>	<i>Laminin (ng/10⁶cell)</i>	<i>Variation %</i>
Control	-	Reference	246.4 +/- 23.0	Reference
Invention product	8.8 ppm	+1	360.0 +/- 13.4	+46%; $p < 0.01$
	17.6 ppm	-9	602.9 +/- 24.7	+145%; $p < 0.01$
Verbascoside	8.8 ppm	-18	193.2 +/- 22.3	-22%; $p < 0.01$
	17,6ppm	-25	202.1 +/- 10.4	-18%; $p < 0.01$

At the tested doses, the product according to the invention stimulates strongly and in a dose-dependent manner the laminin synthesis, whereas verbascoside is inactive. As with fibroblasts, it was observed with keratinocytes a beginning of toxicity at 8.8ppm of verbascoside, which does not appear with the product according to the invention.

b) **Hyaluronic acid**

Table 25: Compared effect between verbascoside and the invention product on hyaluronic acid synthesis on human keratinocytes

<i>HA Keratinocytes</i>	<i>PPG concentration</i>	<i>Cell nb variation (BCA)</i>	<i>Laminins (ng/10⁶cell)</i>	<i>Variation %</i>
Control	-	Reference	4728.7 +/- 537	Reference

Invention product	8.8 ppm	-11	5562.8 +/- 203.8	+18%; $p<0.05$
	17.6 ppm	-5	6373.1 +/- 689.6	+35%; $p<0.01$
Verbascoside	8.8 ppm	-25	4907.5 +/- 602.8	+4%; <i>nsd</i>
	17.6 ppm	-27	5477.9 +/- 480	+16%; <i>nsd</i>

At the tested doses (8.8 and 17.6ppm), the product of the invention stimulates in a dose-dependent manner the laminin synthesis. Verbascoside evaluated in parallel has no significant effect. The beginning of toxicity at 8.8ppm of verbascoside is confirmed by another method (BCA).

c) On the reinforcement of the epidermal barrier

5 • Loricin synthesis

Confluent human keratinocytes were contacted with the test product (product according to the invention or verbascoside) in a calcium containing medium, and differentiation was monitored. After 7 days of culture, the layers are rinsed, fixed and loricin synthesis is highlighted by immunofluorescence and quantified by image analysis. A counter staining of nuclei with Hoechst completes the study and weights these results.

Table 26: Compared effect between verbascoside and the invention product on loricin synthesis on human keratinocytes

<i>Loricin IMF</i>	<i>PPG concentration</i>	<i>Cell nb variation (HXT)</i>	<i>Variation % (AFU/10⁶cell)</i>
Control	-	Reference	Reference
Invention product	17.6 ppm	-37	+670%; $p<0.01$
Verbascoside	17.6 ppm	-23	-56,4%; <i>nsd</i>

The decrease in cell number is classic after a pro-differentiating contact with a product and does not reflect cytotoxicity of this product, but rather a lower adhesion of the differentiated cells to the support.

At the tested dose, the product according to the invention strongly stimulates the synthesis of loricin, and therefore the differentiation of the keratinocytes, whereas verbascoside is inactive.

E) Galenics

Different formulations are described below. Additional cosmetic active ingredients, in support and/or in complement of the activity of the active ingredient according to the invention if necessary can be added to the appropriate phase according to their hydrophobic or hydrophilic nature. These ingredients can be of any class according to their(s) function(s), place of application (body, face, neck, chest, hands, hair, eyelashes, eyebrows, body hair, etc.), final desired effect and target consumer, for example anti-aging,

anti-wrinkle, moisturizing, anti-wrinkle, firming, anti-glycation, slimming, soothing, myo-relaxing, anti-redness, anti-stretch marks, etc.

Active ingredient according to the invention used in the galenic formulas given below: 20% in weight with regard to the total weight of the composition of fresh whole meristematic cells (corresponding to 1% of dried cells), the ingredient having a final content of 0.1% of total phenylpropanoid glycosides (expressed in plantamajoside) in an physiologically acceptable excipient mixture of glycerin, xanthan gum thickener and citric acid to regulate pH if necessary.

1) Cream form

Product	%	INCI name
Phase A		
H ₂ O	Qsp100	Water
Optasense G83	0.30	Carbomer
Phase B		
Brij S2-SS-(RB)	0.40	Steareth-2
Brij S10-SO-(RB)	1.20	Steareth-10
Crodafos CES-PA-(RB)	4.00	Cetearyl Alcohol & Dicetyl Phosphate & Ceteth-10 Phosphate
Crodacol CS90-PA	1.50	Cetearyl Alcohol
Laurocapram	2.50	Laurocapram
BRB CM 56	2.00	Cyclopentasiloxane & Cyclohexasiloxane
Crodamol OSU-LQ-(RB)	7.00	Diethylhexyl Succinate
Phase C		
Glycerin	4.00	Glycerin
Octanediol	0.50	Caprylyl Glycol
Phase D		
Phenoxyethanol	qs	Phenoxyethanol
Phase E		
Potassium sorbate	qs	Potassium Sorbate
Phase F		
H ₂ O	4.00	Water
NaOH 30 %	0.40	Sodium Hydroxide
Phase G		
Active ingredient according to the invention	2.00	
Phase H		
Perfume	0.10	Fragrance

Protocol: Weigh phase A and let swell for 30 minutes under stirring. Heat to 75°C using a water bath.

Weigh phase B and heat to 75°C using a water bath. Mix well. Weigh and melt phase C until dissolved at 45°C. Add phase D in cooled phase C. Pour phase C+D in phase A under striring v=500 rpm. Mix well. Pour phase B in previous phase under staro stirring v=1000 rpm. Mix well. Extemporaneously, add phase E, mix well. Add phase F, mix wel. Add phase G under 45°C, mix well, for 1 hour. Add phase H, mix well.

Examples of ingredients that can be added to this formulation:

- MATRIXYLTM synthe'6: peptide-based anti-wrinkle ingredient marketed by Sederma (WO2010/082175) which helps repair skin damage caused by aging. 3% by weight can be added for example at the end of the formulation.
- 5 • MATRIXYLTM3000: peptide-based anti-wrinkle ingredient marketed by Sederma (WO2005/048968) comprising two matrikines Pal-GHK and Pal-GQPR, which in synergy helps repairing skin damages caused by aging. 3% by weight can be added for example at the end of the formulation.

2) Serum form (fluid emulsion)

Product	%	INCI name
Phase A		
H ₂ O	Qsp100	Water
Potassium sorbate	0.10	Potassium Sorbate
Phase B		
Glycerin	5.00	Glycerin
Phenoxyethanol	qs	Phenoxyethanol
N-Hance HP40	0.10	Hydroxypropyl Guar
Vivapur CS 032	1.00	Microcrystalline Cellulose & Xanthan Gum
Keltrol CG-SFT	0.10	Xanthan Gum
Phase C		
Cithrol PG3PR-LQ-(RB)	1.00	Polyglyceryl-3 Polyricinoleate
Cithrol GMIS 40-LQ-(MV)	3.00	Glyceryl Isostearate
Keteol V 5	1.50	Squalane Vegetal
Crodamol GTCC-LQ-(MV)	2.00	Caprylic/Capric Triglyceride
Crodamol ISIS-LQ-(MV)	2.00	Isostearyl Isostearate
Perfume	0.10	Fragrance
Phase D		
Active ingredient according to the invention	2.00	

- 10 Protocol: Weigh phase A and homogenize under propeller stirring v=300 rpm. Weigh and mix phase B. Add phase B into phase A under propeller stirring v=300 rpm. Let homogenized for 1 hour. Weigh and mix phase C. Add phase C to phase A+B under high propeller agitation v=1500 rpm. Add phase D under propeller stirring v=300 rpm.

Examples of ingredients that can be added to this formulation:

- 15 • RESISTEMTM: anti-aging marketed by Sederma (WO2012/104774), helping the skin to build its own anti-aging defense system, based on an extract obtained by cell culture of *Globularia cordifolia* plant. 2% can be added at the end of the formulation in phase E.
- RIGINTM: active marketed by SEDERMA (WO2000/433417) improving the elasticity and firmness of the skin, increasing hydration and smoothing the skin. 3% by weight of this ingredient
- 20 may for example be added at the end of the formulation.

3) Night cream form

Product	%	INCI name
Phase A		
H ₂ O	Qsp100	Water
Potassium sorbate	0.10	Potassium Sorbate
Phase B		
Keltrol CG-SFT	0.30	Xanthan Gum
Supercol GF	0.15	Guar Gum
Glycerin	4.00	Glycerin
Phenoxyethanol	0.80	Phenoxyethanol
Phase C		
Crodacol C90-PA-(RB)	1.50	Cetyl Alcohol
Crodamol SS-PA-(RB)	2.00	Cetyl Esters
Syncrowax HRC (R)(PAST)	1.50	Tribehenin
Span 60-PW-(MV)	1.00	Sorbitan Stearate
Span 40-PW-(MV)	1.50	Sorbitan Palmitate
Cithrol GMIS 40-LQ-(MV)	2.50	Glyceryl Isostearate
Crodamol ISIS-LQ-(MV)	8.00	Isostearyl Isostearate
Seaton Almond Oil organic-NA07413	2.00	Prunus Amygdalus Dulcis Oil
Crodamol GTCC-LQ-(MV)	8.00	Caprylic/Capric Triglyceride
Crodamol GTIS-LQ-(MV)	2.00	Triisostearin
Coviox T 90	0.10	DI alpha Tocopherol
Phase D		
Active ingredient according to the invention	2.00	/
Phase E		
Perfume	0.10	Fragrance
Phase F		
H ₂ O	1.00	Water
Lactic acid	0.03	Lactic Acid

Protocol: Weigh phase A. Weigh phase B and mix, homogenize well. Add phase B into phase A under staro stirring v=500 rpm for 30 min. Heat phase A+B at 75°C using a water bath. Weigh and mix phase C. Heat phase C at 75°C using a water bath. Pour phase C into phase A+B under very strong staro agitation v=1500 rpm, then v=1000 rpm. Add phase D to the previous phase, under staro stirring v=900 rpm, below 45°C. Add phase E to the previous phase under staro stirring. Adjust the pH to 5.80 +/- 0.10 with phase F.

Examples of ingredients that can be added to this formulation:

- PRODIZIATM: active ingredient marketed by Sederma (WO2013/046137) fighting the signs cutaneous fatigue caused by glycation and glycooxidation. 2% may be added between phase D and phase E under staro stirring v=900 rpm, below 35 ° C.
- RESISTEMTM: mentioned above. 2% can be added at the end of the formulation in phase E.
- MEIRITAGETM: anti-ageing active ingredient based on three plant extracts (*Astragalus membranaceus* (Huang Qi), *Bupleurum falcatum* (Chai Hu) and *Atractylodes macrocephala* (Bai Zhu), which improves the uniformity and radiance of skin.

4) Eye contour gel form

Product	%	INCI name
Phase A		
H ₂ O	Qsp100	Water
Potassium sorbate	0.10	Potassium Sorbate
Phase B		
Glycerin	3.00	Glycerin
Phenoxyethanol	qs	Phenoxyethanol
Keltrol CG-SFT	0.15	Xanthan Gum
Supercol GF	0.15	Cyamopsis Tetragonoloba (Guar) Gum
Crodesta F-50-PW-(RB)	0.90	Sucrose Distearate
Crodesta F-160-PW-(RB)	0.30	Sucrose Stearate
Phase C		
Keteol V 5	3.00	Squalane
Crodamol IPIS-LQ-(MV)	2.00	Isopropyl Isostearate
Crodamol GTCC-LQ-(MV)	1.00	Caprylic/Capric Triglyceride
Phase D		
Active ingredient according to the invention	2.00	
Phase F		
Perfume	0.10	Fragrance

Protocol: Weigh phase A and heat at 75 ° C using a water bath under staro stirring v=700 rpm. Weigh and mix phase B. Add phase B to phase A slowly stirred at 75°C using a water bath. Thoroughly mix for 30 minutes v=700rpm. Weigh and heat phase C at 75°C using a water bath. Pour phase C into phase A+B under staro stirring v =4000rpm. Cool under staro stirring v=750rpm (outside bath). Add phase D, homogenize v=2000rpm below 45°C. Add phase E, homogenize.

Examples of ingredients that can be added to this formulation:

- BEAUTIFEYE™: active ingredient marketed by Sederma ; an association of an *Albizia julibrissin* and of darutoside extracted from *Siegesbeckia orientalis*, demonstrates a lifting action on sagging upper eyelids. 3% can be added at the end of the formulation before phase E.
- MATRIXYL synthe'6™: mentioned above. 3% in weight can be added at the end of the formulation.
- HALOXYL™: active ingredient for dark circles marketed by Sederma (WO2005/102266). Haloxyl™ combines Pal-GHK and Pal-GQPR matrikines with N-hydroxysuccinimide (NHS) and the chrysin flavonoid. The Pal-GHK and Pal-GQPR reinforce firmness and tone of the eye contour; chrysin and N-hydroxysuccinimide activate the elimination of blood origin pigments responsible for the color of dark circles but also of the local inflammation. 3% by weight of this ingredient may be added at the end of the formulation.
- EYELISS™: active ingredient marketed by Sederma (WO2003/068141) that helps prevent against the appearance of bags under the eyes. It combines three components: hesperidin methyl chalcone reducing capillary permeability, Valyl-Tryptophan (VW) dipeptide which promotes lymphatic

circulation and Pal-GQPR lipopeptide that improves firmness, elasticity and reduces inflammation. 3% by weight of this ingredient may be added at the end of the formulation.

5) Tightening cream form

Product	%	INCI name
Phase A		
H ₂ O	Qsp100	Water
Potassium sorbate	0.10	Potassium Sorbate
Phase B		
Glycerin	5.00	Glycerin
Phenoxyethanol	qs	Phenoxyethanol
N-Hance HP40	0.40	Hydroxypropyl Guar
Keltrol CG-SFT	0.50	Xanthan Gum
Phase C		
Span 40-PW-(MV)	1.50	Sorbitan Stearate
Span 60-PW-(MV)	1.00	Sorbitan Palmitate
Crodamol IPIS-LQ-(MV)	3.00	Isopropyl Isostearate
Phase D		
Active ingredient according to the invention	2.00	
Phase E		
Skin Tightener	10.00	
Phase F		
Perfume	0.10	Fragrance

Protocol: Weigh phase A and put it under propeller stirring v=300rpm. Weigh and mix phase B. Add phase B into phase A under propeller stirring v=800rpm, mix well. Heat phase A+B at 75°C using a water bath. Weigh and mix phase C and heat at 75°C using a water bath. Add phase C into phase A+B, under strong propeller stirring v=1000rpm, mix well. Add phase D below 45°C to the previous phase. Add phase E in the previous phase, mix well. Add phase F, below 35°C, mix well.

Examples of ingredients that can be added to this formulation:

- 10 • CALMOSENSINETM: soothing active for sensitive skins marketed by Sederma (WO1998/07744) comprising the Tyr-Arg lipo-dipeptide. It reduces discomfort feelings.
- PHYTOTONINETM: active marketed by Sederma comprising a synergistic combination of three vegetable actives, flavonoids from *Arnica montana* flowers, saponins from rhizomes of *Polygonatum multiflorum* (Solomon's Seal) and proanthocyanidins from *Cupressus sempervirens* cones (Cypress); clearly improves the appearance of "blotchy skin".
- 15 • CHROMOCARETM: anti-age active ingredient marketed by Sederma (WO2010/119423); an association of an extract of *Rabdosia rubescens* rich in oridonine and an extract of *Siegesbeckia orientalis* rich in darutoside; evens and rejuvenates the complexion.

6) Alcoholic gel form

Product	%	INCI
Phase A		
H ₂ O	Qsp100	Water
Phase B		
Keltrol CG-SFT	0.30	Xanthan Gum
Supercol GF	0.30	Guar Gum
Butylene Glycol	3.00	Butylene Glycol
Phenoxyethanol	qs	Phenoxyethanol
Phase C		
Potassium sorbate	0.10	Potassium Sorbate
Phase D		
96%Alcohol	10.00	Ethanol
Phase E		
Active ingredient according to the invention	2.00	

Protocol: Prepare phase A. Weigh and mix phase B. Weigh phase C. Add phase B into phase A under propeller stirring v=125rpm. Pour phase C into phase A+B under propeller stirring v=125rpm. Add phase D, homogenize under propeller stirring v=500rpm. Add phase E, homogenize.

5 Examples of ingredients that can be added to this formulation:

- AQUALANCETM: osmo-protector moisturising active ingredient marketed by Sederma (WO2009/104118) comprising homarine and erythritol.
- NG Birch SapTM: raw sap from birch sapwood; skin toning and moisturizing marketed by Sederma.

10 7) UV protection cream form

Product	%	INCI name
Phase A		
H ₂ O	Qsp100	Water
Potassium sorbate	0.10	Potassium Sorbate
Phase B		
Veegum Ultra	0.80	Magnesium Aluminum Silicate
Keltrol CG-SFT	0.50	Xanthan Gum
Phase C		
Crodesta F160-PW-(RB)	1.00	Sucrose Stearate
Arlacel 2121-FL-(MV)	3.50	Sucrose Cocoate & Sorbitan Stearate
Phase D		
Crodamol IPIS-LQ-(MV)	5.00	Isopropyl Isostearate
Crodamol GTCC-LQ-(MV)	5.00	Caprylic/Capric Triglyceride
Prisorine 3505-LQ-(GD)	5.00	Isostearic Acid
Phase E		

Solaveil CT-300-LQ-(WD)	8.00	Titanium Dioxide & Caprylic/Capric Triglyceride && Polyhydroxystearic Acid & Stearic acid & Alumina
Solaveil CZ-300-LQ-(WD)	2.00	Zinc Oxide & Caprylic/Capric Triglyceride & Polyhydroxystearic Acid & Isostearic Acid
Phase F		
Butylen glycol	4.00	Butylene glycol
Phenoxyethanol	qs	Phenoxyethanol
Phase G		
Active ingredient according to the invention	2.00	
Phase H		
Perfume	0.10	Fragrance
Phase I		
H ₂ O	8.00	Water
L-Lactic acid	0.80	Lactic acid

Protocol: Weigh phase A. Weigh phase B and sprinkle in phase A under staro stirring v=300rpm until complete dispersion. Heat phase A+B at 80°C. Heat using a water bath, under staro stirring v=300rpm. Add phase C into phase A+B under staro stirring v=600rpm at 80°C using a water bath. Weigh and heat phase D at 80°C using a water bath. Pour phase D in the previous phase under staro stirring v=3500rpm. Weigh phase E and heat using a water bath at 80°C. Add phase E in the previous phase under staro stirring v=2000rpm, outside of the water bath. Extemporaneously, add phase F in the previous phase under staro stirring v=2000rpm. Add phase G below 45°C, mix well. Add phase H, mix well. Adjust the pH to 5.60 +/- 0.1 with phase I, below 35°C under staro agitation v=1000rpm, let homogenize for 1 hour.

10 Examples of ingredients that can be added to this formulation:

- VENUCEANETM: active marketed by Sederma (WO2002/066668) comprising a *Thermus thermophiles* biotechnological extract, that prevents visible signs of photo-aging (spots, wrinkles, dryness ...), protects cell structures from damages caused by UV and strengthens skin integrity. 5% can be added for example after phase G.
- 15 • MELASLOWTM: active marketed by Sederma, promotes complexion lightening and age spot depigmentation (extract of Japanese Mandarin *Citrus reticulata* Blanco var. *unshiu*).

8) Night balm form (fluid emulsion)

Product	%	INCI name
Phase A		
H ₂ O	Qsp100	Water
Potassium sorbate	0.10	Potassium Sorbate
Phase B		
Glycerin	10.00	Glycerin
Phenoxyethanol	qs	Phenoxyethanol
Keltrol CG-SFT	0.20	Xanthan Gum
Phase C		
Syncrowax HRC-PA-(RB)	3.00	Tribehenin

Stearic acid	1.50	Stearic Acid
Span 20-LQ-(SG)	1.80	Sorbitan Laurate
Span 80-LQ-(RB)	1.00	Sorbitan Oleate
Crodamol GTCC-LQ-(MV)	4.00	Caprylic/Capric Triglyceride
Keteol V 5	2.00	Squalane
Phase D		
Active ingredient according to the invention	2.00	
Phase E		
Perfume	0.10	Fragrance

Protocol: Weigh phase A. Weigh and homogenize phase B. Add phase B into phase A under propeller stirring v=400 rpm, for 30 minutes. Heat phase A+B to 75°C using a water bath. Weigh phase C and heat to 75°C using a water bath. Add phase C into phase A+B under staro stirring v=2000rpm. Cool under staro stirring v=600rpm. Add phase D at 45°C. Add phase E below 35°C.

5 Examples of ingredients that can be added to this formulation:

- REVIDRATE™: active marketed by Sederma (WO 2011/086532) that in particular improves the cohesion of the epidermis and its hydration. 2% of this ingredient may for example be added to phase C of the formulation.

9) Body and hand cream form

Product	%	INCI name
Phase A		
H ₂ O	Qsp100	Water
Sodium benzoate	qs	Sodium Benzoate
Potassium sorbate	qs	Potassium Sorbate
Phase B		
Glycerin	4.00	Glycerin
Keltrol CG-SFT	0.30	Xanthan Gum
Supercol GF	0.15	Guar Gum
Phase C		
Cithrol GMS 40 SE-PA-(SG)	3.50	Glyceryl Stearate
Crodacol CS90-PA-(RB)	0.50	Cetearyl Alcohol
Crodamol SS-PA-(RB)	1.00	Cetyl Ester
Cithrol GMIS 40-LQ-(MV)	1.00	Glyceryl Isostearate
Crodamol GTIS-LQ-(MV)	2.00	Triisostearin
Crodamol GTCC-LQ-(MV)	2.00	Caprylic/Capric Triglyceride
Crodamol ISIS-LQ-(MV)	4.00	Isostearyl Isostearate
Phase D		
Shea butter unsaponifiables	1.50	Insaponifiables de Beurre de Karité
Phase E		
Active ingredient according to the invention	2.00	
Phase F		
Perfume	0.10	Fragrance
Phase G		
H ₂ O	1.00	Water
Lactic acid	0.07	Lactic Acid

Protocol: Weigh phase A and mix, under propeller stirring $v=300\text{rpm}$. Weigh and mix phase B. Slowly add phase B into phase A under propeller stirring $v=300\text{rpm}$, mix well for 30 min. Heat phase A+B at 75°C using a water bath. Weigh and mix phase C. Heat phase C at 75°C using a water bath. Weigh phase D. Add phase D to phase C. Add phase C+D to phase A+B, under staro stirring $v=2500\text{rpm}$, outside the bath. Add phase E to the previous phase, mix well. Add phase F in the previous phase, mix well. Adjust the pH to $\text{pH} = 5.50 \pm 0.10$ with phase G.

Shea butter is an ingredient with nourishing and protective properties for the treatment of skin damaged by the environment.

Examples of ingredients that can be added to this formulation:

- 10 • INTENSLIMTM: slimming active ingredient marketed by Sederma (WO2013/105048) corresponding to a synergistic combination of extracts obtained by *Globularia cordifolia* plant cell culture, *Zingiber zerumbet Smith* titrated in zerumbone and vegetable caffeine obtained by supercritical CO_2 extraction.
- 15 • JUVINITYTM: active marketed by Sederma (WO 2011/125039) reducing signs of aging on the face and neckline, smoothing wrinkles, densifying and restructuring the dermis.
- RENOVAGETM: global anti-aging active ingredient marketed by Sederma (WO2006/120646). Can be added in particular in place of shea butter.
- Bio-BustylTM: active marketed by Sederma based on peptides (PalGHK and PalVGVAPG) and a bacterial filtrate having a global action on firmness and tone of the bust.

20

F) In vivo Evaluations

A cream prepared according to galenic example 1 (see section F) above) was used for these evaluations.

Principle

25 The assessment of the efficacy of the cream according to the invention was conducted on a panel of 56 mature women aged of mean age 62.5 (52 to 75) in whom the back of the hand and crow's feet, two areas visibly altered by ageing , were monitored using various complementary methods:

- Measurement of elasticity and firmness parameters using Cutometry;
- Measurement of dermal integrity using a Reviscometer®;
- 30 • Measurement of the thickness of the dermis using ultrasonography;
- Measurement of the crow's feet relief by imprint analysis;
- Measurement of epidermal thickness using *in vivo* confocal laser scanning microscopy (Vivascope®); and
- Measurement of hand pigmentation by analyzing images on standardized photographs and
- 35 adhesives.

Protocol**Specific inclusion criteria**

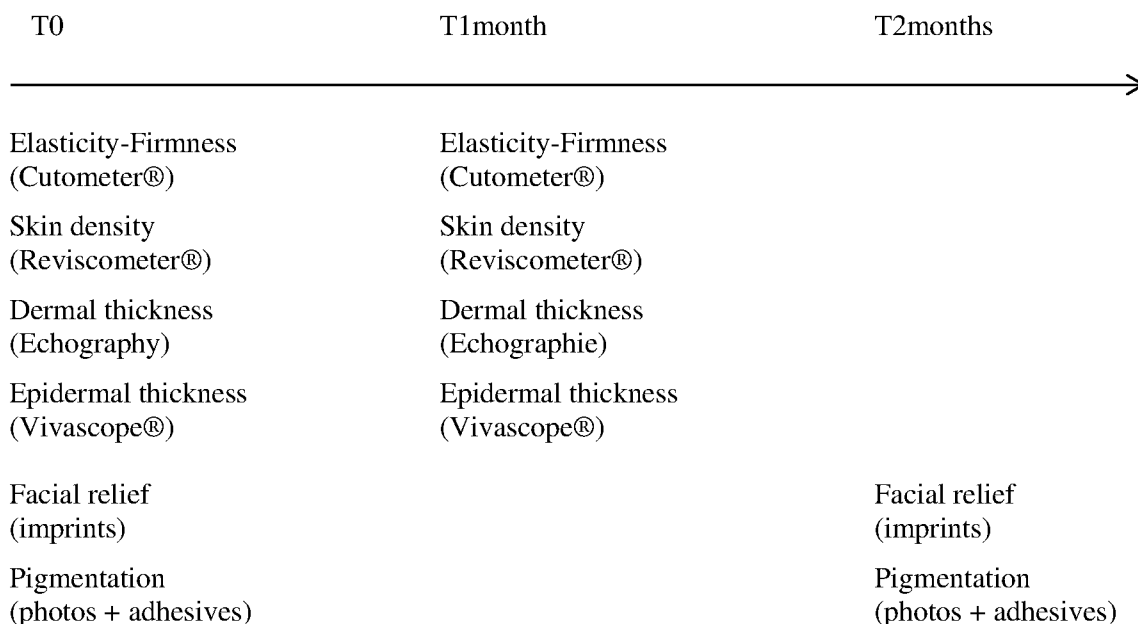
The volunteers were required to present visible signs of ageing on the tested body parts, such as senescence spots, sagging hand skin and/or deep crow's feet. Since not all volunteers presented all three parameters in an optimal way at the time (sometimes with differences between two sides), all tests were not done on each volunteer, and subpopulations were created to have consistent panels for each test.

Volunteers were required to observe a two-week washout period of no cosmetic treatments and maintain hormonal constancy (no change in contraception or hormone replacement/treatment) for 3 months prior to the test and during the test. Volunteers were required to use only the cosmetics provided during the study.

Study types and durations

The study was carried out as a single-blind study on the back of the hand and the face. The cream according to the invention and the placebo cream, used contralaterally, were applied and massaged in twice daily for a period of 2 months.

The study synopsis can be summarized according to the diagram below.



Statistical testing was performed on paired series using the Student's *t* test or, if needed a Wilcoxon signed-rank test.

1) **Improvement of dermal quality**

• **Improvement of eslasticity and firmness of hand skin**

A Cutometer® MPA580 (Courage & Khazaka, Germany) was used to measure the elasticity and firmness of the skin of the hand. This device, which is widely used to assess the effects of cosmetic products, measures the deformation of an area of skin subject to mechanical stress (suction) and the

skin's ability to return to its original shape (i.e. recuperate). The 2-mm probe was used with 500mbar of negative pressure. The figure 1 of the drawings show an example of skin deformation graph thus obtained.

Of the available parameters, those for which age-related skin changes on the back of the hand were clearly demonstrated in the cosmetic literature were chosen: Firmness (Ur/Ue), Elasticity (Ur/Uf) and Recuperation (Ua/Uf). Since these parameters are ratios, they have the advantage of being independent from variations in skin thickness.

Table 27: Variation in elasticity and firmness after applying of the cream of the invention or its placebo (26 volunteers, n= 3 measurements)

	Invention product					
	Recuperation rate Ua/Uf		Firmness Ur/Ue		Elasticity Ur/Uf	
	T0	T1	T0	T1	T0	T1
Mean	0.517	0.584	0.272	0.356	0.190	0.233
+/- SD	0.075	0.080	0.061	0.098	0.043	0.059
Variation vs.T0 (%)		12.9%		30.8%		22.4%
Significance		<i>p<0.01</i>		<i>p<0.01</i>		<i>p<0.01</i>
Significance vs. placebo		<i>p<0.05</i>		<i>p<0.05</i>		<i>p<0.05</i>

	Placebo					
	Recuperation rate Ua/Uf		Firmness Ur/Ue		Elasticity Ur/Uf	
	T0	T1	T0	T1	T0	T1
Mean	0.536	0.536	0.293	0.321	0.205	0.210
+/- SD	0.074	0.075	0.065	0.099	0.043	0.059
Variation vs.T0 (%)		0%		9.5%		2.4%
Significance		<i>nds</i>		<i>nds</i>		<i>nds</i>

nds: non-significant difference

Significant improvement in the three key parameters of Recuperation, Firmness and Elasticity was observed compared to the placebo (*p<0.05*). The increase in 1month is significant (*p<0.01*) on the side who received the invention product; it reaches respectively +12.9%, +30.8% and +22.4%, the effect of the placebo being negligible and non-significant.

- **Measurement of the skin density with a Reviscometer®**

The Reviscometer® RV 600 (Courage & Khazaka, Germany) measures the propagation time for a sound wave emitted at the surface of the dermis and epidermis. This parameter is called *Resonance Running Time* (or RRT). The skin probe of the instrument contains an acoustic transmitter separated from the receiver by a distance of 2mm. The probe does not penetrate at more than 0.5mm, it is thus possible to study the qualities of the dermis. The speed with which the sound propagated in a material depends on the density and tension of the material. The device measures RRTs at different angles by rotating the probe on the surface of the skin. This provides a mean propagation time (mean RRT),

which is described as diminishing with age on the hand, on other sites and when there is tissue breakdown. The mean RTT therefore is reflective of the quality of all the fibers; it diminishes concomitantly with the density of the tissue, contrary to the RRT max. The RRT max increases following this breakdown. The waves move along the strongest fibers, which are those that are least rapidly broken down, and are accentuated.

The studies showed that the mean RTT diminished with age on the hand. The cream according to the invention, or contra-laterally its placebo, was applied on the hand of the volunteers. A mean RTT was calculated using 19 successive rotations of the probe on the skin at T0 and after 1 month of application. 3 measurements were performed each time on each site.

10 **Table 28:** Variation of the mean RTT after applying of the cream of the invention or its placebo (26 volunteers, n=19 measurements x 3)

RRT (ms)	Invention cream		Placebo	
	T0	T1month	T0	T1month
Mean	84.4	113.9	82.8	90.9
+/- SD	41.3	58.1	34.4	36.9
variation vs.T0 (%)		35%		9.8%
Significance		<i>p<0.01</i>		<i>nsd</i>
Significance vs. placebo		<i>p<0.05</i>		

The results demonstrate a significant increase (25.2% (*p<0.05*)) in mean RTT following treatment with the treatment with the cream of the invention compared to the placebo side. This indicates improved skin density.

15 **• Measurement of dermal thickness using ultrasonography**

Ultrasonography, or echography, is a well-established technique for measuring skin thickness. When ultrasound waves encounter tissue in the human body, they are reflected and send back a signal, or an “echo”. The intensity of an echo is translated by the ultrasound into digital grayscale, creating a reliable anatomical image of the area being explored.

20 The instrument used was a Derascan™ C (Cortex Technology, Denmark), which has a 20-Mhz frequency probe that provides images that are 12mm in width and 15mm in penetration. A successive sequence of images provides 5 representative images for study.

Table 29: Variation of dermis thickness after applying of the product of the invention or its placebo (29 volunteers, n=5 measurements).

Thickness (μm)	Invention cream			Placebo		
	T0	T1month	T2months	T0	T1month	T2months
Mean	925	958	973	926	923	940
+/- SD	86	80	97	105	87	106
Variation vs. T0		+33μm	+49μm		-3μm	+14μm
Variation vs. T0 (%)		3.6%	5.2%		-0.3%	1.5%
Significance		<i>p<0.01</i>	<i>p<0.01</i>		<i>nsd</i>	<i>nsd</i>
Significance vs. placebo		<i>p<0.05</i>	<i>p<0.05</i>			

These results demonstrate that applying of the cream of the invention produced a significant mean increase in dermis thickness of 33 μ m (+3.6%; $p<0.01$). In parallel, the site that received the placebo did not demonstrate any variation. Comparing both sites showed that the cream of the invention was more effective ($p<0.05$). Advantageously, the effect is amplified at T2months to reach +5.3% ($p<0.05$ vs. placebo).

Therefore, the improvement in elasticity and firmness is correlated with the increase of the dermal thickness observed here and the increase in density measured using the Reviscometer®. This translates into a qualitative improvement in the skin, and in the dermal fibers in particular.

- **Crow's feet smoothing**

An analysis of the overall smoothing effect of the relief was performed on the crow's feet on each side of the face at the beginning and the end of the application period (2months). Negative imprints were digitalized using the shadow technique with a Visioline VL650 station® (Monaderm, Monaco). The obtained image was then analyzed using the specific Mountains Map® software (Digital Surf, France) in order to quantify the parameter that best determines overall smoothing effect: complexity. The complexity of a surface is the ratio between the raised area (the area occupied if the raised portion of an area is pulled to make it flat) and the non-raised area. A percentage is obtained, and if it diminishes, this indicates smoothing of the relief.

Table 30: Variation of complexity after applying the cream of the invention or its placebo (29 volunteers, n=1 measurement)

	Invention cream		Placebo	
	Complexity (%)		Complexity (%)	
	T0	T2months	T0	T2months
Mean	28.86	25.42	28.24	27.44
SD	9.84	8.53	9.69	8.02
Variation vs. T0 (%)		-11.9%		-2.8%
Significance		$p<0.01$		<i>dns</i>
Significance vs. placebo		$p<0.01$		

After two months of applying the cream of the invention, there was a very significant smoothing ($p<0.01$) of nearly -12% in complexity. The decrease on the placebo side was negligible and non-significant (-3%). Comparing both sides showed that the effects were better with the cream of the invention ($p>0.01$).

2) Increase of the epidermal thickness

- **Measurement of the epidermal thickness using confocal laser scanning microscopy**

It is known that the epidermis thins with age, especially on the hand. To assess this parameter, the epidermis was analyzed using confocal laser scanning microscopy with a Vivascope® 3000 (Mavig, Germany), which has a portable, maneuverable probe.

The light emitted by the instrument (830nm wavelength) is reflected differently in the skin depending on the refraction index of the structures it hits (keratin, melanin and micro-cellular structures, as well as collagen, will appear in white on a greyscale digital image). The Vivascope® enables to “lighten” a specific point in the skin (focalization of illumination) and in parallel precisely detects the returning light (focalization of the detection). This instrument is called “Confocal” for “conjugate focal planes”. This enables to perform a real non-invasive biopsy of the skin in real time to study healthy skin, its pigmentation, its ageing, its photoageing and the thickness of its different layers.

During the study, vertical acquisitions spaced 2.6µm apart were performed to view the complete epidermis. The measurement of the thickness of the live epidermis between the end of the *stratum corneum* and the beginning of the papillae was then performed manually on several sites of a given image acquisition to take into consideration potential local variations.

The thicknesses calculated in this way were consistent with the specific hand sites mentioned in the literature.

Table 31: Variation of epidermal thickness after applying the cream of the invention or its placebo (22 volunteers, n=2 measurements)

	Invention cream		Placebo	
	T0	T1month	T0	T1month
Mean	31.00	35.75	31.18	32.95
SD	7.98	8.04	7.23	6.81
Variation in µm vs. T0		4.75µm		1.77µm
variation vs. T0 (%)		15.3%		5.7%
Significance		<i>p</i> <0.01		<i>dns</i>
Significance vs. placebo		<i>p</i> =0.06		

The analysis of the results demonstrates an increase in live epidermis thickness after applying the cream of the invention. Remarkably this significant increase (*p*<0.01) reaches +4.75µm (+15%) compared to T0. The placebo treated site only demonstrated a non-significant increase of +1.77µm (+5.7%), and comparison with the cream of the invention shows a significant difference of 3µm (approximately 10%) in its favor (*p*=0.05).

Furthermore, if only the treatment responders (72%) are considered, the results demonstrate a much greater increase on the invention cream side (+8.21µm) compared to the placebo side (+3.03µm), generating a significant difference (*p*<0.05) of 5µm (approximately 17%).

This regeneration of epidermal cellular layers is coupled with the vitalizing effect already observed in the dermis.

- **Reduction in pigmentation, analysis by photographs**

At T0 and after 2 months of application, photographs were taken with a mounted, high-definition digital camera using specific lighting and a system for positioning the volunteer's hands. Hand position, photographic parameters and lighting were standardized and controlled so that they could be reproduced over time.

Hyperpigmentation areas with or without spots were selected. The L^* or lightness parameter was analysed in each photograph using Colorskin™ software to identify increases in L^* . However, since skin thickness does not change from one extremity of the scale to the other, a constant of $L^*=30$ was selected to provide a more physiological scale.

- 5 Table 32: Variation of the lightness of the skin L^* after applying the product of the invention or its placebo (26 volunteers, n= 1 measurements)

Lightness L^*	Invention cream				Placebo			
	Area with senescence spot		Area without senescence spots		Area with senescence spot		Area without senescence spots	
	T0	T2	T0	T2	T0	T2	T0	T2
Mean	14.5	19.1	27.1	30.4	16.1	19.6	27.4	29.2
SD	5.7	5.4	3.3	3.6	5.6	5.5	3.8	3.7
Variation vs. T0		4.6		3.3		3.5		1.8
variation vs. T0 (%)		31.7%		12.2%		21.7%		6.6%
Significance		$p<0.01$		$p<0.01$		$p<0.01$		$p<0.01$
Significance vs. placebo		$p<0.05$		$p<0.05$				

- These results demonstrate that applying the cream of the invention significantly lightens pigmented areas. Thus, the L^* variation observed for areas with spots and those without spots was respectively 31.7% and 12.2% compared to T0, which represents more and better lightening ($p<0,05$) than what was observed for the placebo site (21.7% and 6.6% respectively).

It should be noted, that the depigmentation effect is observable both for areas with spots and for the areas around the spots, leading to an overall lightening effect.

- **Decrease of spots, adhesive analysis**

- Samples of *stratum corneum* were taken using adhesives (Dsquam®; Cuderm™) and were colored using the “Fontana Masson” method to reveal the melanin that remained in the *stratum corneum*. Standardized photographs were taken and analyzed to quantify this residual pigmentation by expressing it as the percentage represented by melanin of the non-pigmented residual. Given the intricacy of the method, only 14 volunteers had samples taken.

- 20 Table 33: Variation in the quantity of melanin found in the *stratum corneum* 2months after applying of the product of the invention or its placebo (14 volunteers, n= 1 measurement)

	Invention cream		Placebo	
	Melanin (%)		Melanin (%)	
	T0	T2months	T0	T2months
Mean	6.337	4.703	5.818	4.951
SD	1.5	1.4	1.1	1.1

Variation vs. T0 (%)		-26%		-15%
Significance		$p<0.01$		$p<0.01$
Significance vs. placebo		$p<0.01$		

A decrease is thus observed in the quantity of melanin on both sides, but the decrease is greater on the side that received the cream with 2% of the product of the invention. Comparing both sites shows that the cream of the invention was more effective (-11%; $p<0.01$). Therefore, these results confirm the results obtained by photographing both hands.

CLAIMS

1. Production method of *in vitro* meristematic cells of *Plantago lanceolata* comprising phenylpropanoid glycosides using a stabilized cell line, wherein said cell line is IRB PL3 cell line.
- 5 2. Method according to claim 1, characterized by the following steps:
 - from said IRB PL3 *Plantago lanceolata* cell line, producing a critical pre-biomass by successive pre-cultures and increasing sizes;
 - produce a biomass of said meristematic cells in a bioreactor using said pre-biomass and a suitable culture medium; and
 - 10 - separating said biomass enriched in phenylpropanoid glycosides from said culture medium and recovering said meristematic cells.
3. Method according to claim 1 or 2, wherein said bioreactor production step comprises an elicitation step performed by modifying the culture medium.
4. Method according to claim 3, wherein said elicitation step is performed by modifying the
15 nutrients.
5. Method according to anyone of claims 1 to 4, wherein said biomass from the reactor is collected between 7 to 21 days of culture.
6. Method according to anyone of claims 1 to 5, wherein said biomass from the reactor is collected between 10 to 14 days of culture.
- 20 7. Cell line of *Plantago lanceolata* with IRB PL3 reference.
8. Meristematic cells of *Plantago lanceolata* obtained with the IRB PL3 cell line or according to the method of anyone of claims 1 to 6.
9. Cells according to claim 8, comprising plantamajoside as phenylpropanoid glycoside main component.
- 25 10. Cellular extract obtained from cells according to claim 8 or 9.
11. Extract according to claim 10, characterized in that it performed by a cell lysis, then a centrifugation step followed by a filtration, in order to recover the inside of the cells and to eliminate the cell walls.
12. Composition comprising meristematic cells of *Plantago lanceolata* according to claim 8 or
30 9, or a cellular extract according to claim 10 or 11, in a physiologically acceptable medium.
13. Composition according to claim 12, wherein the physiologically acceptable medium is an hydrophilic matrix in which said cells are in suspension.
14. Composition according to claim 12 or 13, comprising a thickener and/or subjected to high pressure homogenization.
- 35 15. Cosmetic composition according to anyone of claims 12 to 14, comprising at least 0.1% of phenylpropanoid glycosides.

16. Use of the cells according to claim 8 or 9, of the cellular extract according to claim 10 or 11, or the composition according to anyone of claims 12 to 15, for a non therapeutical cosmetic treatment.
17. Use according to claim 16 for an anti-ageing treatment of skin and appendages.
- 5 18. Use of meristematic cells of *Plantago lanceolata* or a cellular extract of said cells, or use according to claim 16 or 17, to densify dermis, strengthen dermal-epidermal junction, thicken epidermis and/or provide anti-glycation defense means to the skin.
19. Use according to anyone of claims 16 to 18, to decrease expression of one or more miRNAs selected from miRNAs 29, 21, 196a, 30a, 34a, 152 and 181a.
- 10 20. Use of meristematic cells of *Plantago lanceolata* or cellular extract of said cells, or cells according to claims 8 or 9, or the cellular extract according to claims 10 or 11, or the composition according to anyone of claims 12 to 15, for a nutraceutical application.
21. Use according to claim 20, to strengthen intestinal wall.
22. Meristematic cells of *Plantago lanceolata* or cellular extract of said cells, or cells according to claims 8 or 9, or the cellular extract according to claims 10 or 11, or the composition according to anyone of claims 12 to 15, for a dermatological medical treatment.
- 15 23. Cells, cellular extract or composition according to claim 22, for treating pigmentation disorders, inflammatory disorders (such as atopic dermatitis, psoriasis, acne), bullous dermatoses, disorders involving disorders in the synthesis of collagen, elastin of hyaluronic acid and/or dry skin, rosacea and/or ichthyosis.
- 20

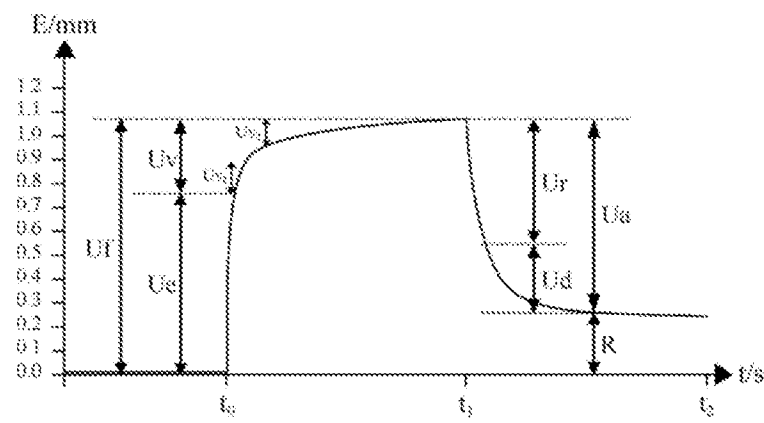


FIG 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/060404

A. CLASSIFICATION OF SUBJECT MATTER

INV. A01H4/00 A61K36/68 C12N5/04 C12N15/04
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A01H A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 319 914 A1 (I R B ISTITUTO DI RICERCHE BIOTECNOLOGICHE S R L [IT]) 11 May 2011 (2011-05-11) cited in the application	1-23
Y	the whole document ----- -/--	1-23



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

1 July 2014

Date of mailing of the international search report

08/07/2014

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2014/060404

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BUDZIANOWSKA ANNA ET AL: "Phenylethanoid glucosides from in vitro propagated plants and callus cultures of <i>Plantago lanceolata</i> ", PLANTA MEDICA, vol. 70, no. 9, September 2004 (2004-09), pages 834-840, XP002719166, ISSN: 0032-0943 abstract page 834, left-hand column, line 2 - line 7 page 839, left-hand column, line 2 - line 4	1-23
Y	----- RENATA DAWID-PAC: "Medicinal plants used in treatment of inflammatory skin diseases", ADVANCES IN DERMATOLOGY AND ALLERGOLOGY, vol. 3, 1 January 2013 (2013-01-01), pages 170-177, XP55074848, ISSN: 1642-395X, DOI: 10.5114/pdia.2013.35620 Plantaginis lanceolatae folium/herba - ribwort plantain leaf/herb.; page 74	1-23
A	----- DI PAOLA R ET AL: "Teupolioside, a phenylpropanoid glycosides of <i>Ajuga reptans</i> , biotechnologically produced by IRBN22 plant cell line, exerts beneficial effects on a rodent model of colitis", BIOCHEMICAL PHARMACOLOGY, ELSEVIER, US, vol. 77, no. 5, 1 March 2009 (2009-03-01), pages 845-857, XP025941476, ISSN: 0006-2952, DOI: 10.1016/J.BCP.2008.11.010 [retrieved on 2008-11-25] the whole document -----	1-23

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Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2319914	A1	11-05-2011	NONE
