International Journal of Cosmetic Science, 2021, **43**, 165–180 doi: 10.1111/ics.12678

Overall renewal of skin lipids with Vetiver extract for a complete anti-ageing strategy

Morgane De Tollenaere*, Emilie Chapuis*, Laura Lapierre*, Marine Bracq*, Jane Hubert†, Carole Lambert*, Jérome Sandré‡, Daniel Auriol*, Amandine Scandolera* and Romain Reynaud*

*Givaudan France SAS, Research and Development, Argenteuil, France, †NatExplore SAS, Bezannes, France and ‡Chirurgien plasticien et esthétique, Polyclinique de Courlancy, Reims, France

Received 3 August 2020, Revised 20 November 2020, Accepted 26 November 2020

Keywords: chemical analysis, claim substantiation in vivo/in vitro, skin barrier, skin physiology/structure

Abstract

OBJECTIVE: Skin lipids are essential in every compartment of the skin where they play a key role in various biological functions. Interestingly, their role is central in the maintenance of hydration which is related to skin barrier function and in the skin structure through adipose tissue. It is well described today that skin lipids are affected by ageing giving skin sagging, wrinkles and dryness. Thereby, developing cosmetic actives able to reactivate skin lipids would be an efficient ant-ageing strategy. Due to the strong commitment of our scientists to innovate responsibly and create value, they designed a high value active ingredient named here as Vetiver extract, using a ground-breaking upcycling approach. We evidenced that this unique extract was able to reactivate globally the skin lipids production, bringing skin hydration and plumping effect for mature skin.

METHOD: In order to demonstrate the global renewal of lipids, we evaluated the lipids synthesis on cutaneous cells that produce lipids such as keratinocytes, sebocytes and adipocytes then on Reconstructed Human Epidermis and skin explants. We evaluated the expression of proteins involved in ceramides transport and barrier cornification. We then evaluated hydration and sebaceous parameters on a panel of mature volunteers.

RESULTS: We firstly demonstrated that Vetiver extract induced sebum production from human sebocytes cells lines but also improved its quality as observed by the production of specific antimicrobial lipids. Secondly, we demonstrated that Vetiver extract was able to restore skin barrier with the increase of skin lipids neosynthesis on Reconstructed Human Epidermis and skin explants. We also evidenced that Vetiver extract stimulated the lipids transport and epidermal cornification. Finally, Vetiver extract showed a significant effect on adipogenesis and maturation of adipocytes at in vitro and ex vivo models. We confirmed all these activities by showing that Vetiver extract improved sebum production and brought hydration through an increase of lipids content and their conformation. Vetiver extract induced an improvement of skin fatigue and a plumping effect by acting deeply on adipose tissue.

CONCLUSION: In conclusion, we developed an active ingredient able to bring anti-ageing effect for mature skin by a global increase of skin lipids.

Correspondence: Morgane De Tollenaere, Skin Biology Scientist, Givaudan Active Beauty, Route de Bazancourt, Pomacle 51110, France. e-mail: morgane.de_tollenaere@givaudan.com

Résumé

OBJECTIF: Les lipides de la peau sont essentiels dans chaque compartiment de la peau où ils jouent un rôle clé dans diverses fonctions biologiques. Il est intéressant de noter que leur rôle est central dans le maintien de l'hydratation, liée à la fonction de barrière cutanée, mais aussi dans la structure même de la peau, par le biais du tissu adipeux. Il est bien décrit aujourd'hui que les lipides de la peau sont affectés par le vieillissement, ce qui entraîne un relâchement de la peau, des rides et une sécheresse. Ainsi, le développement d'actifs cosmétiques capables de réactiver les lipides de la peau serait une stratégie efficace de lutte contre le vieillissement. En raison de l'engagement fort de nos scientifiques à innover de manière responsable et à créer de la valeur, ils ont conçus un ingrédient actif à forte valeur ajoutée, appelé ici extrait de Vétiver, en utilisant une approche révolutionnaire de « up-cycling ». Nous avons démontré que cet extrait unique était capable de réactiver globalement la production de lipides de la peau, apportant une hydratation de la peau et un effet repulpant pour les peaux matures.

MÉTHODES: Afin de démontrer le renouvellement global des lipides, nous avons évalué la synthèse des lipides sur les cellules cutanées qui produisent des lipides tels que les kératinocytes, les sébocytes et les adipocytes, puis sur un modèle d'Epiderme Humain Reconstruit et les explants de peau. Nous avons évalué l'expression des protéines impliquées dans le transport des céramides et la kératinisation de la barrière cutanée. Nous avons ensuite évalué l'hydratation et les paramètres sébacés sur un panel de volontaires matures.

RÉSULTATS: Nous avons tout d'abord démontré que l'extrait de Vétiver induit la production de sébum à partir de lignées cellulaires de sébocytes humains mais améliore également sa qualité comme l'indique la production de lipides antimicrobiens spécifiques. Ensuite, nous avons démontré que l'extrait de Vétiver était capable de restaurer la barrière cutanée grâce à l'augmentation de la néosynthèse lipidique sur un modèle d'Epiderme Humain Reconstruit et sur des explants de peau. Nous avons également démontré que l'extrait de Vétiver stimulait le transport des lipides et la kératinisation de l'épiderme. Enfin, l'extrait de Vétiver a montré un effet significatif sur l'adipogenèse et la maturation des adipocytes dans des modèles *in vitro* et *ex vivo*. Nous avons confirmé à l'échelle clinique toutes ces activités en montrant que l'extrait de Vétiver améliorait la production de sébum et apportait une hydratation grâce à une augmentation de la teneur en lipides ainsi qu'une modification de leur conformation. L'extrait de Vétiver a induit une

amélioration de la fatigue cutanée et un effet repulpant en agissant en profondeur sur le tissu adipeux.
CONCLUSION: En conclusion, nous avons développé un ingrédient actif capable d'apporter un effet anti-âge aux peaux matures par une augmentation globale des lipides de la peau.

Introduction

Chrysopogon zizanioides, more commonly known as Vetiver, is a perennial grass widely cultivated in tropical regions. The plant, known to stabilize soil and limit erosion, is studied for phytoremediation and its roots contain an essential oil that is renowned in the fine fragrance and cosmetic industries. The essential oil has been traditionally used in South Asia as an anti-inflammatory, antimicrobial and healing agent (non-exhaustive list) [1,2]. Moreover, Chomchalow in his review evoked the ability of the essential oil to moisturize and rejuvenate the skin.

Adding value to each part of a cultivated plant and to its by-products has become a concern for each industry with the aim of producing sustainable products. With this in mind, a cosmetic ingredient was developed from hydrodistilled roots of Vetiver that are generally considered to be a coproduct that is burned or composted. Considering the properties of Vetiver essential oil, we evaluated in this study the biological function of this extract and especially its effect on the skin lipids. Indeed, the skin lipids are produced to ensure the integrity of the epidermal barrier, to promote skin hydration and to participate in the filling of wrinkles through the plumping of adipocytes. The boosting of skin lipids may provide an efficient anti-ageing strategy.

Lipids and barrier function

The lipids present in the skin play an important role in barrier function and thus skin hydration.

Their renewal is performed by the differentiation mechanism from the basal layer to the cornified layer of the epidermis. The cornified layer, also named *stratum corneum,* contains the vast majority of skin lipids which are mainly composed of ceramides, cholesterol and long-chain free fatty acids [3,4]. These lipids are transported through various pathways which can be vesicular dependent or independent [5]. Polar ceramides are synthetized in the endoplasmic reticulum (ER) and their transport from ER to the Golgi apparatus requires a specific protein called CERT (ceramide transport protein) or COL4A3BP [3,6]. CERT is a member of the steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) protein family. It possesses a stereospecific binding site to ceramides and an N-terminal region that binds phosphatidylinositol 4 monophosphate to the Golgi apparatus [7,8]. In the Golgi apparatus, the ceramides and the other lipids produced by keratinocytes are introduced in newly formed vesicles more commonly known as lamellar bodies. They are then released into the extracellular space at the *stratum corneum* by exocytosis [6]. The lamellar bodies deliver lipids, proteins and antimicrobial peptides but also enzymes that transform lipid precursors and lead to desquamation.

The *stratum corneum* is made of cohesive corneocytes containing a cornified cell envelope and an enriched lipid matrix. The cornified cell envelope is essentially composed of structural proteins such as loricrin, involucrin, envoplakin, desmoplakin, elafin, cornifins and periplakin [4]. The stability of the envelope depends on the quantity of these proteins and their degree of cross-linking [9]. This cross-linking is driven by the transglutaminase-1 protein [4]. It provides a scaffold for extracellular lipid lamellae and together they shape the cornified envelope that provides skin hydration and impermeability against xenobiotics and exogenous factors [3,4,10].

Recently, a new secreted protein from the stratified epithelium was described in the literature: the dermokine. The expression of the human dermokine gene codes for four various isoforms with three of them (α, β and γ) that are secreted as peptides in the epidermis. The β and γ isoforms are specifically expressed by the granular keratinocytes with an intracellular localization between the granular and cornified layers that supposes a secretion through the lamellar bodies [11,12]. Previous studies proved that dermokine plays a role in cornification and promotes involucrin expression through the regulation of the ERK signalling pathway [11,13]. As such, these studies suggest that dermokine could also be a key factor in lipid–protein interaction involved in envelope formation, similar to involucrin.

Lipids and sebum

A second source of lipids delivered by the sebum has essential abilities, as a result of their hydrolipidic film-forming property [14]. Sebum is released on the skin surface to promote skin protection and hydration. It is produced by sebaceous glands which are mostly associated to hair follicles forming the pilosebaceous unit. More precisely, they are composed of sebocytes which produce lipids during their differentiation to finally release sebum by holocrine secretion. Sebum is composed of various lipids such as triglycerides, wax esters, squalene, glycerol and short and long-chain free fatty acids [9,15,16]. Among the fatty acids produced by the sebaceous glands, lauric acid and sapienic acid exert antimicrobial activity against Gram-positive and –negative bacteria [17,18]. These two antimicrobial lipids (AMLs) may act in synergy with the antimicrobial peptides (AMP), produced by keratinocytes and contribute to the skin's defence. Sebum is also well-known to have a strong antioxidant protective effect against UV irradiation [15].

Lipids and subcutaneous adipose tissue

The remaining source of skin lipids is brought by the third layer of the skin: the adipose tissue. This compartment is well characterized and possesses its own properties. The hypodermis stores energy and brings endocrine and structural support to the dermis layer [19,20]. The particular distribution of fat gives a plumping effect to the skin. The adipose tissue is composed mainly by two types of cells: the pre-adipocytes and adipocytes. The skin structure is provided by the increase of the number of adipocytes through the process of pre-adipocytes differentiation called adipogenesis; and by their maturation called lipogenesis. When the energy requirement is fulfilled, the surplus is deposited in the form of neutral triglycerides inside their uniloculuar lipid droplet [21]. Recently, various papers highlighted the growing role of dermal adipose tissue in skin functions through its involvement on tissue repair, hair regeneration and immune support [22,23]. The mature adipocytes from this compartment may release antimicrobial peptides to fight bacterial infection [23,24].

Lipids and ageing

During the ageing process, an important decrease in lipid content of 30% is observed in the *stratum corneum*, which is directly correlated to ageing-mediated-delayed barrier renewal [3]. Unfortunately, this

phenomenon can be also accelerated by various factors, such as cold weather and detergent application, and all of them lead to skin dryness [9,25]. A delayed barrier renewal results in inefficient corneodesmosomes degradation, a decrease in natural moisturizing factor (NMF) and ceramides level, and an imbalance in skin composition [9,25]. This results in the appearance of wrinkles.

Similarly to the lipids from the skin barrier, the production of lipids from sebum is significantly affected by ageing. The rate of sebum secretion decreases after 20 years of age by 23% per decade for men and 32% per decade for women [26]. As a consequence, skin dryness seems to be related to a decrease in protection against microbiota [27].

On top of that, the subcutaneous adipose tissue is also impacted with age. The skin on the face is particularly affected by loss and redistribution of fat [20,28].The atrophy of the adipose tissue aggravates the apparition of wrinkles through the thinning and sagging of the skin [19,22,28]. The phenomenon could be explained by the decrease of metabolic responsiveness of the cells, leading to a decrease of pre-adipocytes replication and differentiation [20,29,30].

To prevent this cosmetic issue and fill facial wrinkles, autologous facial fat grafts are often used in aesthetic surgery [19,31]. In some literature, the idea of stimulating adipogenesis as facial filler alternative is mentioned [28]. Continuing with this hypothesis, we considered that boosting the lipid synthesis inside adipocytes combined with an improvement of skin surface lipids appeared to be an effective anti-ageing strategy.

The main objective of this study was to prove whether the global reactivation of skin lipids synthesis using Vetiver extract could influence the skin appearance. The final objective expected was to provide skin hydration and plumping effect for an anti-ageing strategy. In addition, we have demonstrated that the improvement of skin lipids could bring antimicrobial protection through AML synthesis.

Material and methods

Chemicals and reagents

Acetonitrile, toluene, ethyl acetate and methyl-*tert*-butyl ether were purchased from Carlo Erba Reactifs SDS (Val de Reuil, France). Acetic acid and formic acid were purchased from Merck (Darmstadt, Germany). DMSO-*d6* was purchased from Eurisotop (Saint Aubin, France). Deionized water was used to prepare all aqueous solutions.

Plant extract preparation

Chrysopogon zizanioides roots were collected in Haiti and hydrodistilled for oil extraction. The resulting exhausted roots were dried naturally, crushed and extracted in water with a plant/solvent ratio of 1/20 (w/v). The extract was obtained after root washing and extraction at 60°C for 6 hours. Solid/liquid separation was then achieved by filtration and the extract was concentrated under vacuum. For centrifugal partition chromatography purpose, the extract was freeze-dried after concentration by rotary evaporator.

A specific standardized extract titrated in zizanoic acid was used for all the biological evaluations.

Extract fractionation by centrifugal partition chromatography

The dry extract of *Chrysopogon zizanioides* was fractionated by Centrifugal Partition Chromatography (CPC) on a laboratory-scale column of 303 mL capacity (FCPE300®; Rousselet Robatel Kromaton, Annonay, France) with the biphasic solvent system methyl-*tert*-butyl ether/acetonitrile/water (4/1/5, v/v). The aqueous phase was used as the stationary phase. The extract (1.53 g) was dissolved in 20 mL of aqueous phase and 5 mL of organic phase and loaded into the CPC column. The organic phase was pumped in the ascending mode at 20 mL min^{-1} (Knauer Preparative 1800 V7115 pump; Berlin, Germany) for 70 minutes and then in the descending mode for 8 minutes for column extrusion. Column rotation speed was set at 1200 rpm. Fractions of 20 mL were collected over the whole experiment by a Pharmacia Superfrac collector (Uppsala, Sweden).

Fractions were spotted on Merck TLC plates coated with silica gel 60 F254 and developed with ethyl acetate, toluene, acetic acid and formic acid (20/80/11/11, v/v). After inspection at 254 and 366 nm, the plates were sprayed with vanillin/H_2SO_4 and heated to 100°C for 2 minutes. Fractions of similar TLC profiles were combined, resulting in a final series of 13 fractions (F1 to F13) which were dried and re-suspended in 600 µL of DMSO-*d6*.

NMR analyses and metabolite identification

NMR analyses were performed on a Bruker Avance AVIII-600 spectrometer (Karlsruhe, Germany) equipped with a TXI cryoprobe. Spectra were manually phased, baseline corrected using the TOPSPIN 3.2 software (Bruker) and calibrated on the central resonance of DMSO-*d6* (δ 39.80 ppm). Rapid identification of known compounds (dereplication) was realized according to the method published by Hubert and colleagues [32]. Briefly, the absolute intensities of all peaks detected in the ^{13}C NMR spectra of F1-F13 were automatically collected and binned across the spectra of the fraction series using a locally developed computer script. The obtained table was submitted to Hierarchical Clustering Analysis (PermutMatrix 1.9.3; LIRMM, Montpellier, France). This HCA step consists in measuring the similarities between groups of NMR peaks that are present in successive spectra of the fraction series (based on the CPC elution profile) and thus that presumably belong to single molecules. These groups of correlated NMR peaks are visualized as 'chemical shift clusters' on a HCA heat map given in Figure 1. The 13 columns of the heat map correspond to the ^{13}C NMR spectra of the fraction series, and the rows are chemical shift bins of the detected NMR peaks. The higher the intensity of ^{13}C NMR signals, the brighter the colour on the map. For metabolite identification, each ^{13}C NMR chemical shift cluster obtained from HCA was manually submitted to an in-house database comprising the structures and predicted chemical shifts of low molecular weight natural products (prediction tools from ACD/NMR Workbook Suite 2012 software, ACD/Labs, Ontario, Canada). In parallel, a literature survey was performed to obtain the names and chemical structures of a maximum of metabolites already reported in the species *Chrysopogon zizanoides* ($n \approx 294$). All fractions were also analysed by 2D NMR (HSQC, HMBC and COSY) to confirm or to complement the elucidation of the molecular structures proposed by the database at the end of the dereplication process.

In vitro lipid neosynthesis in RHE

The study was performed using Reconstructed Human Epidermis (RHE). After transfer to air/liquid interface at day 0 (D0), the RHEs were placed in the assay medium (confidential) containing or not containing (for the control) Vetiver extract or the reference

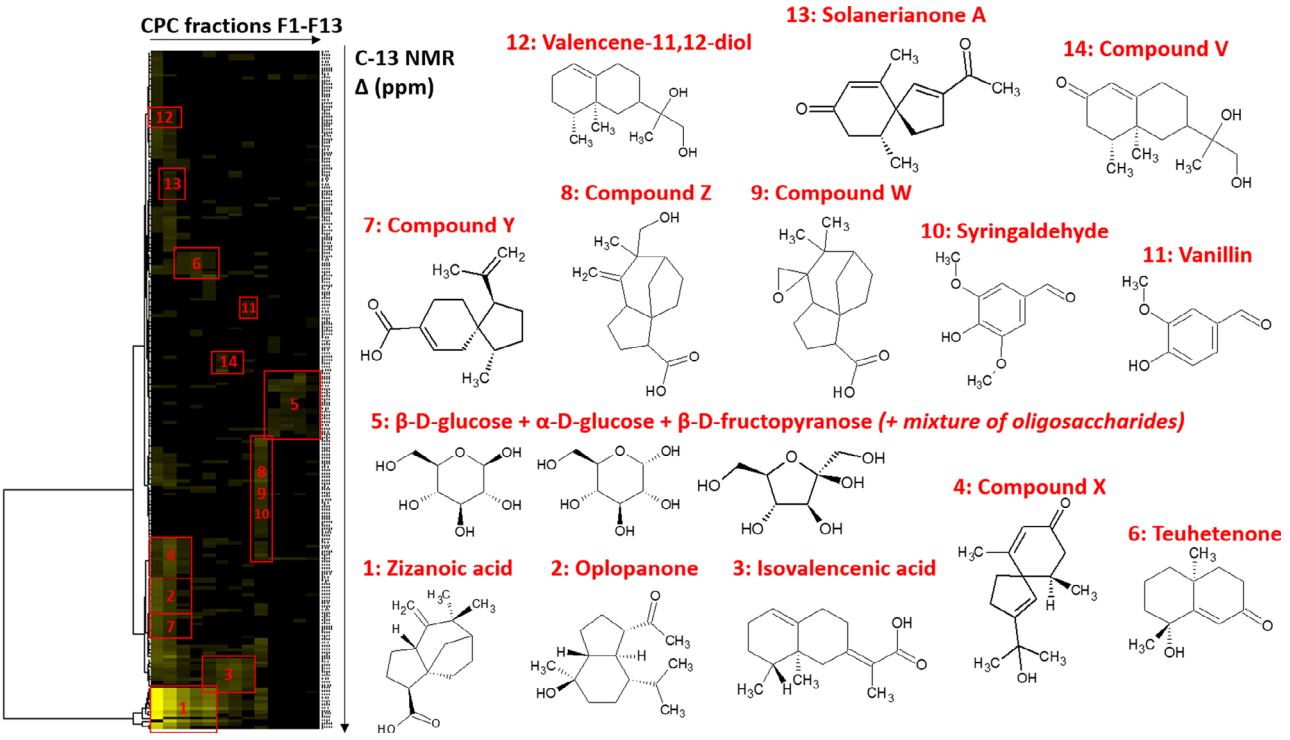

Figure 1 C-13 NMR chemical shift clusters (yellow colour) obtained by applying HCA on CPC fractions of *Chrysopogon zizanioides* extract 1 and the chemical structures of major compounds identified in the extract.

(vitamin C at 200 µg mL^{-1}, Sigma-Aldrich, Saint-Louis, MO, USA). After 3 days of culture (D3), the medium was replaced by the assay medium containing Vetiver extract at 1% (v/v) or the reference and the radioactive tracer, [^{14}C]-acetate (Perkin Elmer, Waltham, MA, USA), was added. After 2 days of incubation (D5), the medium was discarded and replaced by assay medium containing the different treatments (Vetiver extract or reference), and then, RHEs were incubated for 2 additional days (end of the culture at D7). All experimental conditions were performed in triplicate. After incubation, the RHEs were rinsed with a phosphate-buffered saline (PBS) solution and frozen at −80°C until analysis. Then, the RHEs were dissociated and lysed on ice using a 0.5 M perchloric acid solution (Sigma-Aldrich). Lipids contained in the RHEs were extracted using a methanol/chloroform mix according to Bligh and Dyer protocol [33]. Organic phases were dried by evaporation, then deposited on silica plates in order to perform thin-layer chromatography (TLC) using a mobile phase for polar lipid separation (chloroform/methanol/water). The TLC plate was autoradiographed using a laser scanner (Amersham™ Typhoon™; GE Healthcare, Chicago, IL, USA) and the semi-quantitative analysis of the various lipid species was performed by densitometric analysis using the Multigauge version 3.0 software (Fujifilm, Minato, Tokyo, Japan). The standards used for the identification of the different lipid species are described in the Table S1.

In vitro immunofluorescence staining of CERT, dermokine and involucrin

Normal human keratinocytes (NHKs) were seeded at 50,000 cells per well in 24-well plates containing slides beforehand which are collagen-coated (50 µg mL^{-1}) and incubated at 37°C with 5% CO_2. After 48 hours of culture in complete medium (KGM Gold, Lonza, Basel, Switzerland), the cells were stimulated for 5 days with 1.5 mM Ca^{2+} (Alfa Aesar, Haverhill, MA, USA) supplemented with 2% Foetal Bovine Serum (FBS) (Biowest, Nuaillé, France) as a differentiated control or with Vetiver extract at 1% (v/v). The untreated control was the complete medium. After stimulation of the differentiation, the cells were rinsed 2 times with filtered-PBS and then permeabilized with 2% paraformaldehyde (MP Biomedicals, USA) supplemented with 0.5% Triton X-100 during 5 minutes for CERT and dermokine immuno-detections and there was no permeabilization for involucrin immunostaining. The cells were rinsed again with filtered-PBS and fixed for 15 minutes with 2% Paraformaldehyde. After fixation, the cells were rinsed 2 times with filtered-PBS and the non-specific sites were blocked with 3% Bovine Serum Albumin (BSA, Sigma-Aldrich) and 0.1% Tween 20 (Sigma-Aldrich) for 1 hour. The cells were then incubated at 4°C overnight with the primary antibody diluted at 1:100 in 1:10 of blocking solution (3% BSA + 0.1% Tween 20) in a dark and humid atmosphere. The slides were then washed 3 times with PBS and 0.3% Triton X-100 and incubated with the second antibody (Goat anti-rabbit Alexa Fluor 488) for 2 hours at room temperature in a dark and humid atmosphere. Finally, the cells were washed 3 times with PBS and 0.3% Triton X-100, then 2 times with filtered-PBS and mounted with Prolong Diamond with DAPI counterstaining (Fisher Scientific, USA). The specificity of secondary antibodies was checked as negative controls. Three images of each slide were collected with Axio Observer Inverted fluorescence microscope (Zeiss) and were further analysed with the use of ImageJ software.

The antibodies used for the assays are an anti-CERT/COL4A3BP rabbit polyclonal antibody (Thermo Fisher Scientific, PA5-28797), anti-dermokine beta rabbit antibody (ab26830; Abcam, Cambridge, UK) and anti-involucrin rabbit antibody [Sy5] (ab68, Abcam).

In vitro lipid accumulation on sebocytes

Human SEBO662AR sebocyte cell lines were seeded in 96-well plates (50,000 cells/well) and cultured at 37°C with 5% CO_2 for 24 hours in culture medium (K-SFM supplemented with 25 μg mL^{-1} of gentamycin, Thermo Fisher Scientific). The medium was then removed and replaced by assay medium containing or not containing (for the control) the extract at 1% (v/v), and the cells were pre-incubated for 4 hours. Then, the lipogenic mix (containing vitamin C, vitamin D3, insulin and calcium, and no androgens, BioAlternatives, Gençay, France) was added and the cells were incubated. After 3 days of incubation, half of the medium was removed and the treatments were renewed (including lipogenic mix stimulation). A non-stimulated control condition was performed in parallel. All experimental conditions were performed in triplicate, except for stimulated control conditions in sextuplicate. After 4 days of the incubation, the cells were rinsed, fixed and permeabilized. The lipid droplets contained in the cells were then labelled using a specific Bodipy® fluorescent lipid probe (Thermo Fisher Scientific), labelling mainly neutral lipids. In parallel, the cell nuclei were stained using a Hoechst 33258 (bis-benzimide) solution (Sigma-Aldrich). The acquisition of the images was performed using INCell Analyzer™ 1000 (GE Healthcare, Chicago, IL, USA). 10 photos were taken per well for each labelling (×20 objective lens). The labelling was quantified by the measurement of the fluorescence intensity normalized to the total number of cells (integration of numerical data with the Developer Toolbox 1.5 software, GE Healthcare software, Chicago, IL, USA). Results were expressed as fluorescence intensity normalized to the number of cells, % stimulated control and % inhibition. The fluorescence intensity was analysed exclusively in the lipid droplets (image analysis program based on object segmentation). Therefore, non-specific fluorescent background signal, which can be frequently observed in high confluence cultures of SEBO662AR, was not taken into account in the image analysis.

In vitro Antimicrobial Lipid (AML) synthesis using sebocytes

Human SEBO662AR sebocytes were seeded into culture inserts and grown in culture medium (K-SFM supplemented with 25 μg mL^{-1} of gentamycin, Thermo Fisher Scientific) for 48 hours at 37°C with 5% CO_2. After this immersion culture, the cultures were placed at the air–liquid interface (D0) and the medium was replaced by culture medium containing or not containing (for the control) the compounds being tested or a non-androgenic seborrhoeic reference and the cultures were incubated for 7 days with renewal of the culture medium and treatments (Vetiver extract at 1% (v/v) and reference) every 2 to 3 days. All the experimental conditions were performed in triplicate. The 3D tissues were cut, put in microtubes and dry frozen at −80°C. The determination of the content of Free Fatty Acids is performed using a GC (7890A; Agilent, Santa Clara, California, USA) coupled with a MS system (5975C Inert XL EI/CI MSD, Agilent). The extraction of fatty acids was performed using a liquid/liquid extraction (proportions are the propriety of BioAlternatives). Samples were then methylated (FAME) before analysis.

In vitro stimulation of pre-adipocytes differentiation

Pre-adipocytes were seeded in 96-well plates and cultured in culture medium (DMEM (Gibco) supplemented with L-glutamine at 2 mM, penicillin at 50 U mL^{-1}, streptomycin at 50 μg mL^{-1} and Foetal Calf Serum at 10%) for 24 hours. The culture medium was then replaced with culture medium containing or not (control) the extract at 3% (v/v) and the cells were incubated for 13 days. All experimental conditions were performed in n = 3. At the end of the incubation, cells were rinsed and the lipid droplets were labelled using a fluorescent lipid probe (AdipoRed™, Lonza, Switzerland) and the cell nuclei were stained using Hoechst 33342 solution (bis-benzimide, Thermo Fisher Scientific). The acquisition of the images was performed with the INCell Analyzer™ 2200 (GE Healthcare) and 5 photos per well were taken (Objective lens ×20).The labelling was quantified by the measurement of the fluorescence intensity normalized to the total number of cells identified with Hoechst (Integration of numerical data with the Developer Toolbox 1.5, GE Healthcare software).

Ex vivo stimulation of keratinization

Human skin explants with an average diameter of 11 mm were prepared from biopsies coming from three Caucasian women of 69, 54 and 56 years old. The explants were kept in survival in BEM medium (BIO-EC's Explants Medium, BIO-EC, Longjumeau, France) at 37°C in a humid, 5% CO_2 atmosphere. Vetiver extract at 1% (v/v) was topically applied each day on the basis of 2 μL cm^{-2} on explants and spread with a small spatula. The untreated controls did not receive any treatment except the renewal of the medium. The culture medium (1 mL) was refreshed on D1, D4 and D6.

Neutral lipids were stained on the frozen section using a LipidTOX™ green neutral lipid (ref. 34475, Life technologies, Carlsbad, CA, USA).

HCS LipidTOX™ Green neutral lipid stain is used to visualize neutral lipids on the surface of the skin and in the sebaceous glands. Briefly, skin sections were fixed by Ciaccio solution (formalin-potassium bichromate acetic acid fixative), post-chromizated and incubated with LipidTOX™ green neutral lipid diluted at 1/3000 in PBS for 45 minutes at room temperature. Nuclei were counterstained by DAPI diluted 1:2 in Vectashield (VWR, Radnor, Pennsylvania, USA).

The immunostaining was assessed by microscopy observation (green fluorescence). The fluorescence was quantified for each picture by software imaging (ImageJ). The region of interest was delimited and the total fluorescence intensity was calculated then reported to the total area of interest. The specific labelling is in green and the nuclei are in blue using DAPI labelling. The graphics represent the fluorescence intensity averages ± standard error of the mean. The values were compared to the untreated control to express an activating or inhibiting effect.

Ex vivo full-skin lipids accumulation in mature adipocytes

On an abdominal plasty coming from a 60 years old Caucasian woman, 9 explants of full skin (epidermis + dermis + hypodermis) of an average diameter of 11 mm were prepared. The explants were kept in survival in BEM medium (BIO-EC's Explants Medium, BIO-EC, Longjumeau, France) at 37°C in a humid, 5%-CO_2 atmosphere. On Day 0 (D0), 3 explants were collected and cut in 2 parts: half was fixed in a formol-buffered solution and half was

frozen at −80°C. On D0, D1, D4 and D6, Vetiver extract at 3% (v/v) was topically applied on the basis of 2 μL per explants (2mg.cm^{-2}) on explants and spread with a small spatula. The untreated explants did not receive any treatment except the renewal of the medium. Half of the medium (1 mL) was renewed on D1, D4 and D6. On D8, the explants were collected and processed in the same method used at D0. After fixation for 24 hours in buffered formal solution, the samples were dehydrated and impregnated in paraffin using a Leica PEARL dehydration automat (Leica, Wetzlar, Germany). The samples were embedded using a Leica EG 1160 embedding station. 5 μm thick sections were made using a Leica RM 2125 Minot-type microtome, and the sections were mounted on Superfrost® histological glass slides. The microscopical observations were realized using a Leica DMLB or Olympus BX43 microscope. Pictures were digitized with a numeric DP72 Olympus camera with CellD storing software (Olympus, Rungis, France). The control of the cellular viability was performed after staining of paraffinized sections according to Masson's trichrome, Goldner variant. The staining was evaluated by microscopical observation and image analysis of adipocyte size. For all conditions, the pictures have been realized on the best representative zones of the explants. The analysis has been performed on about 300 adipocytes per condition. The surface and diameter of the adipocytes were measured. The adipocytes were pooled in 3 ranges by their equivalent circular diameter (ECD). The ECD is the diameter of a circle that has the same surface of the measured object. It is expressed as μm.

Clinical investigation of skin hydration and lipid conformation

Panel description

A double blind and placebo-controlled clinical evaluation was carried out on 20 women volunteers aged from 50 to 70 years old with a mean age 63 years old (±2). The volunteers were pre-selected according to their leg skin hydration level, which has to be less than 35 by corneometry measurement. All the subjects participating in the study gave their informed consent at the beginning of the study. The selected volunteers applied the products containing Vetiver extract at 2% (v/v) or the placebo on their leg twice daily (morning and evening) for 28 days. At D0 and D28, skin hydration and lipid conformation were analysed by corneometry and Raman spectroscopy, respectively.

Corneometry analysis

The *stratum corneum* hydration causes changing in its electrical characteristics. The *stratum corneum* is like a dielectric corps. Any modification of its hydration statement causes a variation of the electric capacity measured by a condenser. Higher is the hydration, higher is the electric capacity because its dipolar nature increases the electric permittivity of the environment and its conductibility. Measurement was performed by the Corneometer® CM 825 TM (Courage & Khazaka electronics, Köln, Germany). The probe linked to a condenser allows applying all the time the same pressure on the tegument in order to not disturb the measures and obtain good experimental conditions reproducibility.

The measures were done at D0 and D28 in order to perform comparative analysis.

Raman spectroscopy

Lipid conformation was evaluated by Raman spectroscopy by measuring the ratio between C-C *trans* and C-C *gauche* molecular bond. This ratio is associated with a compact state in the lipid packing, whereas a decrease is indicative of a loosening.

The setup included a confocal Raman probe coupled to a dispersive Raman spectrograph. The excitation laser beam was sent to the remote probe via a 5 μm core-diameter fibre and the Raman signal was conveyed to the spectrograph via 100 μm core-diameter fibre. The probe was equipped with a 100X long working distance objective operating in air.

A piezoelectric device made it possible to collect Z Raman profiles by providing axial measurements from the surface down to a defined depth in the skin. A colour video camera integrated in the probe made it possible to visualize the skin surface. This camera is also useful for controlling the focalization of the laser on the skin.

The spectrograph was equipped with a CCD (Coupled Charge Detector) of 1024 × 256 elements cooled by Peltier effect, and an 830 grooves/mm grating which makes it possible to cover a large spectral range from 550 to 3700 cm^{-1} in a single shot acquisition with a spectral resolution of about 6 cm^{-1}. The excitation source was a 660 nm laser diode and the power at sample was fixed at 30 mW in accordance with protection standards for radiation. For protection against laser radiation, protective glasses are used.

The 660 nm excitation was chosen as the optimal compromise between generation of parasitic fluorescence and sensitivity of the CCD camera over the whole spectral range. For skin characterization, the measurement of high wavenumber vibrations is important to access information on water content. For data acquisition, the device was controlled by the Labspec 5 software. The Z profiles consist of in-depth scanning through the skin. Raman spectra were collected at different focus points, from skin surface Z = 0 μm to 30 μm (under the skin surface) with a 3 μm step, leading to a time of 1 minute for a complete profile. In total, we recorded 240 Raman profiles (2 profiles percondition) and each profile comprises 14 spectra. In total, we recorded 3360 spectra. These measurements will be used for the evaluation of lipids (Evaluation of skin barrier function). The pre-processing of spectral data was performed using Matlab 7.2 (The MathWorks Inc., USA). Aberrant profiles (bad S/N (signal/noise) ratio, fluorescence, incomplete profile with a large zero offset, saturation…) were excluded from the database. Each non-aberrant profile was submitted to background corrections, which made it possible to clean up the Raman signal of the skin. These background corrections included smoothing spectra with a 9 mm Savitzky–Golay filter, spikes correction and baseline correction by using a fourth-degree polynomial function, meant to remove the skin fluorescence. Finally, Raman profiles were normalized on the intensity of the entire wavenumber range, with a vector function.

Clinical investigation on sebum production in elderly volunteers

Panel description

A double blind and placebo-controlled clinical evaluation was carried out on 1 group of 30 women volunteers aged from 63 to 70 years with a mean age 67 years old (±2). The volunteers presented low levels of facial sebum. All the subjects participating in the study gave their informed consent at the beginning of the study. The selected volunteers applied the products containing Vetiver extract at 2% (v/v) or the placebo on their face twice daily (morning and evening) for 28 days. At D0 and D28, sebum production was analysed using Sebumeter® on forehead and cheek.

Sebum analysis by Sebumeter®

The frosted film is contained in a cassette. The plastic film is matt and translucent (similar to frosted glass), with a thickness of 100 μm. The measuring head of the cassette has an area of 64 mm^2. A mirror

is placed under the measuring film at about 1 mm. It is connected to the cassette by a spring which ensures a constant pressure between the film and the area that is to be measured. The duration of the measurement is 30 seconds; it is monitored by a clock built into the device. Sebum is determined by introducing the cassette head into the measuring tube. A cell analyses its transparency. The transmitted light represents the sebum contained on the measured zone. A microprocessor calculates the results, which are then displayed on the screen in μg of sebum cm^{-2} of skin.

Clinical investigation on skin biomechanical property and wrinkles

Panel description

A double blind and placebo-controlled clinical evaluation was carried out on 2 groups of 21 women volunteers aged from 52 to 69 years with a mean age 60 years old (±4). The volunteers presented a sagging face and wrinkles on the face. All the subjects participating in the study gave their informed consent at the beginning of the study. The selected volunteers applied the products containing Vetiver extract at 2% or placebo on their face twice daily (morning and evening) for 56 days. At D0, D28 and D56, skin biomechanical properties were measured by cutometry. The skin relief focused on upper lip was analysed by AEVA-HE® (Eotech, Marcoussis, France) at D0 and D56.

Skin biomechanical properties measured by Cutometer®

The assessment of the skin mechanical properties enables to assess the functional state of the elastic structures (elastic fibres, curvature of the connective bundles, wrinkles of the *stratum corneum*) and the viscous-behaving structures (interstitial fluids, internal adherences). The assessment is performed using the Cutometer® MPA 580 (Courage & Khazaka, Köln, Germany) on cheekbone. The measuring principle is based on the suction method. Negative pressure is created in the device and the skin is drawn into the cylindrical aperture (2 mm in diameter) of the probe. Inside the probe, the penetration depth is determined by an optical measuring system. Each suction phase is followed by a relaxing phase. The resistance of the skin to be sucked up by the negative pressure and its ability to return to its original position are displayed as curves at the end of each measurement. From these curves, the parameters can be calculated. During the suction phase, the deformation of the skin by the negative pressure measures first the elastic resistance, then the viscous component, which taken together represent skin firmness. During the relaxation phase, the immediate recovery of the skin measures sheer cutaneous elasticity, whereas the delayed return of the skin to its initial position measures the visco-elastic component. The creep test results that evaluate the mechanical skin properties are not easy to read into so several parameters. In this study, we used the R9 parameter. It represents the effect of fatigue of the skin after several successive aspirations. The more R9 is small, less the effect of fatigue is important. This parameter was measured at D0 and D56.

Wrinkles analysis by AEVA-HE®: focus on upper lip

The AEVA-HE® system measures the effectiveness of a cosmetic product without contact skin contact. For this study, we used the AEVA-HE® system with the 250 sensors in order to measure the depth, length and number of wrinkles. Based on a fringe projection unit using light associated with stereometry, the AEVA-HE® system offers high-resolution 3D scanning. The volunteers were installed on the VisioTOP-500 benches for accurate and stable positioning and re-positioning between the different measuring times.

All the clinical studies used the same formula:

AQUA/WATER, **+/-VETIVERIA ZIZANIOIDES ROOT EXTRACT**, CAPRIC/CAPRYLIC TRIGLYCERIDE, CETEARYL WHEAT STRAW GLYCOSIDES, CETEARYL ALCOHOL, PHENOXYETHANOL, METHYL PARABEN, PROPYL PARABEN, ETHYL PARABEN, DIMETHICONE, FRAGRANCE, HEXYL CINNAMAL, BUTYLPHENYL, METHYLPROPIONAL, CITRONELLOL, ALPHA ISOMETHYL IONONE, HYDROXYISOHEXYL 3-CYCLOHEXENE CARBOXALDEHYDE,SODIUM HYDROXIDE.

Statistical analysis

All results are presented as mean ± standard error of mean of three independent triplicates. A Shapiro–Wilk test was used to verify whether the raw data followed the Gaussian Law. In case of normally distributed data, the mean values were compared using either an unpaired *t*-test (≤2 groups) or One-way ANOVA followed by post-hoc test (≥3 groups). In case of non-normally distributed data, a Kruskal–Wallis test followed by a Mann–Whitney *U* test was used for unpaired data.

In all the case, it was considered as a significant results with $P < 0.1$ with #, $P < 0.05$ with *, $P < 0.01$ with ** and $P < 0.001$ with ***.

Results

Chemical profiling of the *Chrysopogon zizanioides* exhausted root extract

The major constituents of the exhausted root extract of *Chrysopogon zizanioides* were identified using a dereplication method that combines Centrifugal Partition Chromatography (CPC), NMR analyses, and Hierarchical Clustering Analysis (HCA) of NMR spectral data for pattern recognition. CPC fractionation of the extract resulted in the production of 13 fractions of simplified chemical composition, in an increasing order of polarity, and without any loss of material. All fractions were analysed by NMR and ^{13}C NMR spectra were submitted to Hierarchical Clustering Analysis for the classification and visualization of ^{13}C NMR metabolite fingerprints. As illustrated in Figure 1, the major chemical shift clusters coloured in yellow on the heat map correspond to the major metabolites of the extract. With the help of an in-house database containing predicted chemical shift values of natural metabolites, the correlated chemical shifts of the most intense cluster 1 in fractions F1-F8 were assigned to zizanoic acid (ZA). The identification of ZA was confirmed by checking all ^{1}H-^{13}C and ^{1}H-^{1}H correlations in HSQC, HMBC and COSY spectra of F1. By using the same method, the correlated chemical shifts of clusters 2-10 were assigned to oplopanone (cluster 2) detected in F1-F3, isovalencenic acid (cluster 3) detected in F6-F8, compound X (cluster 4) detected in F1-F3, a mixture of glucose and fructose (cluster 5) detected in the most polar fractions F10-F12, teuhetenone (cluster 6) detected in F3-F5, compound Y (cluster 7) detected in F1-F2, compound Z (cluster 8) detected in F9, compound W (cluster 9) detected in F8-F9, syringaldehyde (cluster 10) detected in F9, and the minor compounds vanillin (cluster 11) detected in F8, valencene-11,12-diol (cluster 12) detected in F1-F2, solanerianone A (cluster 13) detected in F2, and compound V detected in F6.

The major compounds identified here in the exhausted root extract of *Chrysopogon zizanioides* are well-known sesquiterpenic constituents of Vetiver essential oil [34], except compounds V, W, Y and Z which have never been described before, and compound X which is reported here for the first time as a natural compound.

Detailed NMR data obtained experimentally for all metabolites identified in extract are given in a Data S1.

Improvement of skin lipids involved in barrier function

In the first part of this study, we were interested to find out whether Vetiver extract was able to reactivate the lipid synthesis in the skin barrier.

Therefore, we used an RHE model to prove the efficacy of Vetiver extract on skin lipids synthesis involved in the skin barrier. The neosynthesis of lipids was analysed by thin-layer chromatography (TLC). Here, we quantified the main lipid components of the epidermal barrier including ceramides and their precursors. We observed that Vetiver extract at 1% significantly induced the synthesis of sphingomyelin, phosphoglycerides, polar ceramides and cerebrosides by +42%, 38% and 32%, respectively, in comparison with untreated condition after 7 days of stimulation (Figure 2). However, the treatment of the RHE with vitamin C at 200 μg mL^{-1}, used here as positive control, resulted in a significant stimulation of polar ceramides and cerebrosides with +68% relative to the untreated condition. In order to confirm the stimulation of lipid synthesis using Vetiver extract, we performed a LipidTox™ staining on human skin explants after 8 days of topical application. We demonstrated that Vetiver extract at 1% significantly increased the neutral lipid content from skin explants by +29.6% (Figure 3). When taken together, these data demonstrated that Vetiver extract

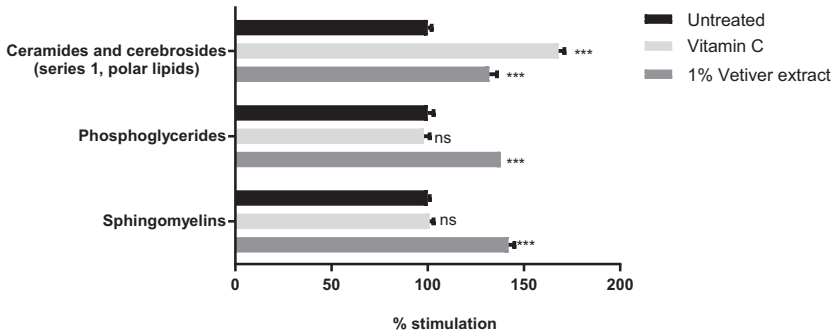

Figure 2 Effect of Vetiver extract on [^{14}C]-acetate incorporation in polar lipids in reconstructed human epidermis. Quantified results are expressed in % of untreated condition. Results of independent triplicate are presented as mean ± SEM. Statistical analysis was performed using ANOVA followed by Bonferroni's multiple comparisons test. The *P*-values presented are expressed in comparison with untreated condition with *** $P < 0.001$.

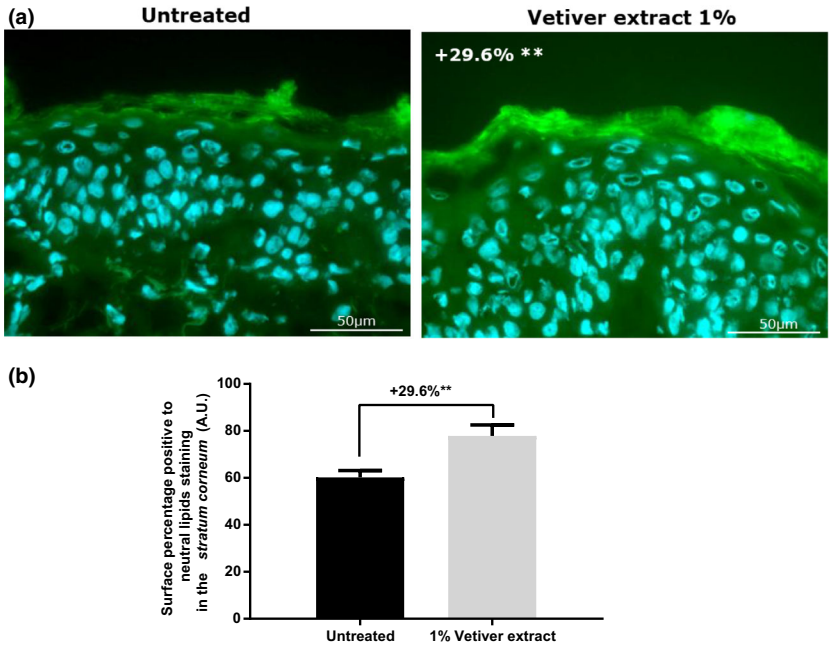

Figure 3 Panel a: Representative pictures of 1% Vetiver extract's effect on neutral lipids synthesis (LipidTox™ staining, magnification ×20). Panel b: Immunofluorescence quantification of the surface percentage positive for neutral lipids staining in the *stratum corneum*. Results of independent triplicate are presented as mean ± SEM. Statistical analysis was performed using Student's *t*-test in comparison with untreated condition with ** $P < 0.01$.

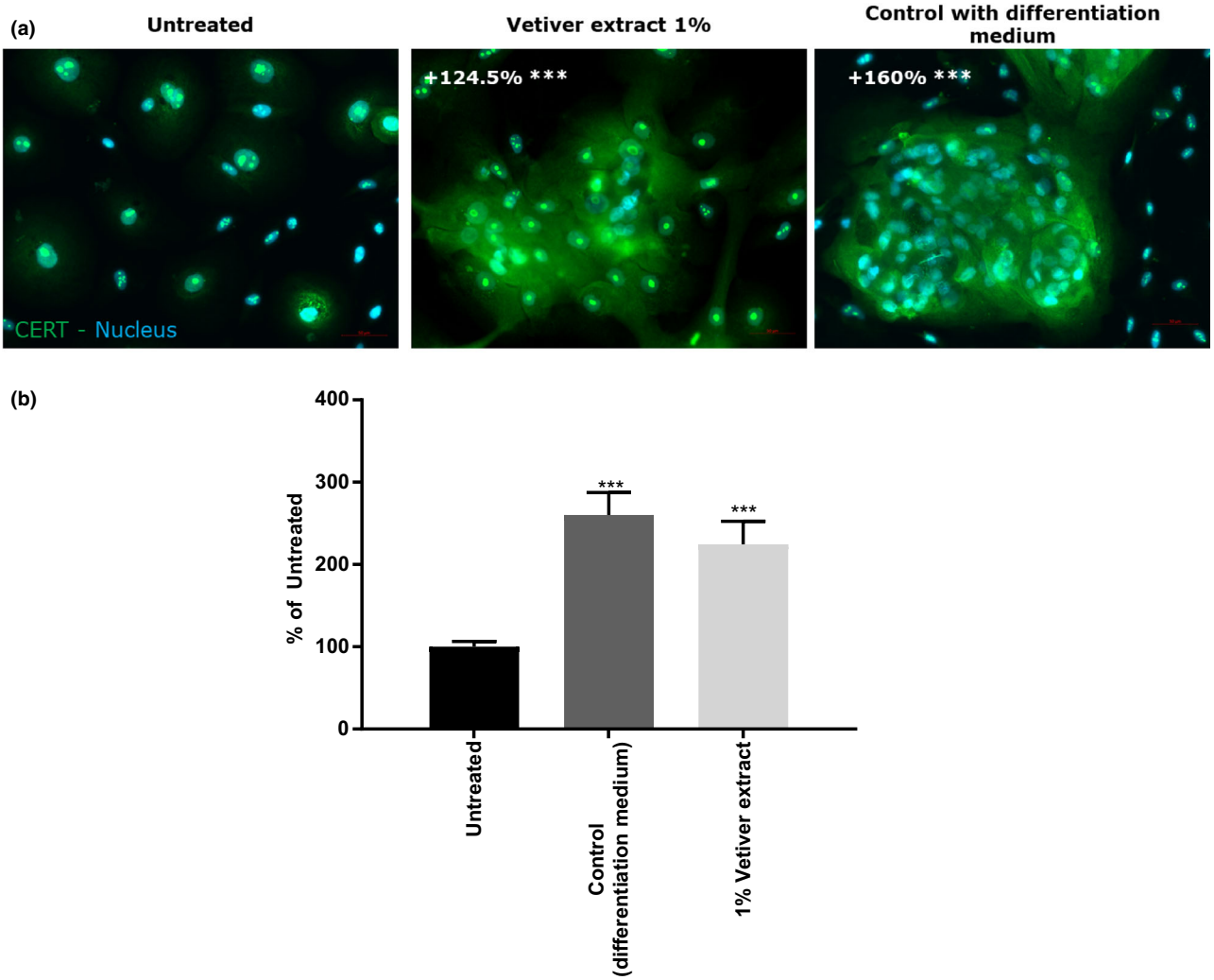

Figure 4 Panel a: Representative images of the impact of Vetiver extract in CERT immunofluorescence staining in NHEKs in comparison to the differentiation medium-treated condition (Magnification ×10). Panel b: Immunofluorescence quantification of the surface percentage positive for CERT staining in NHEKs. Results of independent triplicate are presented as mean ± SEM. Statistical analysis was performed using Kruskal–Wallis test followed by Mann–Whitney *U* test in comparison with untreated condition with *** $P < 0.001$.

was able to stimulate the skin barrier lipids at *in vitro* and *ex vivo* levels.

To go further, we designed an *in vitro* model of differentiation using keratinocytes to mimic the superficial layer of the skin in which the production of lipids is intensive. The cell differentiation was confirmed by the significant increase of CERT expression by +160% and a particular cell morphology which is typically observed in differentiated keratinocytes (Figure 4). Then, we analysed the expression of terminal epidermal differentiation biomarkers such as involucrin and dermokine in order to demonstrate that the activation of lipid transport is well correlated to an improvement in the skin barrier. With the differentiation medium, we demonstrate a significant increase of involucrin and dermokine expressions by +166.7% and +351.5%, respectively (Figure 5), proving the correct cell differentiation and definitively validating our model. Using this model, we wanted to find out whether Vetiver extract was able to promote lipid transport and organization for the formation of the cornified envelope. Therefore, we showed that Vetiver extract at 1% applied for 5 days significantly induced CERT expression by +124.5% (Figure 4) and we evidenced a similar effect from the positive control which is the differentiation medium here. Moreover, we observed that a 5-day treatment with Vetiver extract at 1% significantly stimulated the expression of involucrin by +69% and the expression of dermokine by +90%. These results confirmed that Vetiver extract was able to promote the cell differentiation mechanism through lipid transport and stimulation of two key cornified proteins (Figure 5).

Finally, we carried out a clinical study in order to confirm that Vetiver was able to improve skin barrier and related lipid content and thereby to provide skin hydration. In this study, we compared the efficacy of a cream at 2% Vetiver extract with a placebo cream, after 28 days of twice daily application, on skin hydration

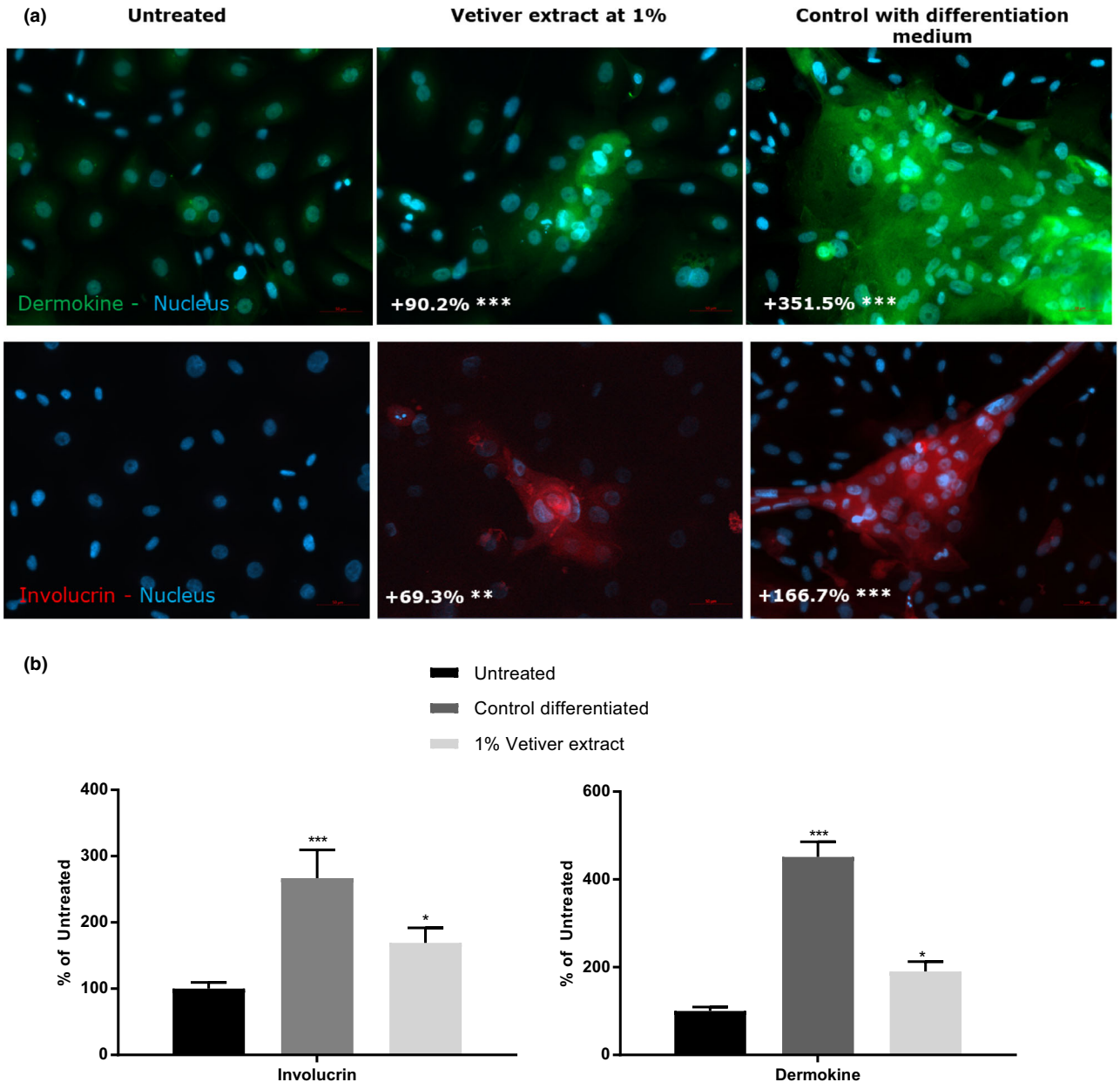

Figure 5 Panel a: Representative pictures of Vetiver extract's effect in dermokine and involucrin immunofluorescence staining in NHEKs (Magnification × 10). Panel b: Immunofluorescence quantification of the surface percentage positive for involucrin and dermokine staining in NHEKs. Results of independent triplicate are presented as mean ± SEM. Statistical analysis was performed using ANOVA followed by Bonferroni's multiple comparisons test for dermokine quantification and Tukey's multiple comparisons test for involucrin quantification. The *p*-values presented are expressed in comparison with untreated condition with ** $P < 0.01$ and *** $P < 0.001$.

and lipid conformation using corneometry and Raman spectroscopy analyses. After 28 days, we observed a significant improvement of skin hydration with Vetiver at 2% by +28.5% compared with D0 as shown in Figure 6. This effect was significantly higher than the placebo with +7% (Figure 6). During the study, we also analysed the lipid conformation by Raman spectroscopy and we showed a significant improvement of lipid conformation by +39.6% relative to D0 with the cream at 2% Vetiver extract while the placebo didn't have any relevant impact. Again, the product at 2% Vetiver extract demonstrated a significant and more effective impact than the placebo after 28 days of application (Figure 7).

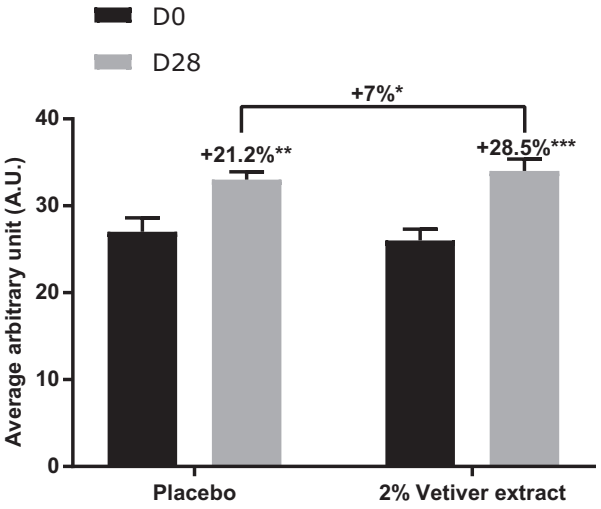

Figure 6 Impact of products containing or not containing Vetiver extract at 2% on skin hydration after 28 days of application analysed by corneometry. Results are presented as mean ± SEM. Statistical analysis was performed using Student's *t*-test in comparison with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

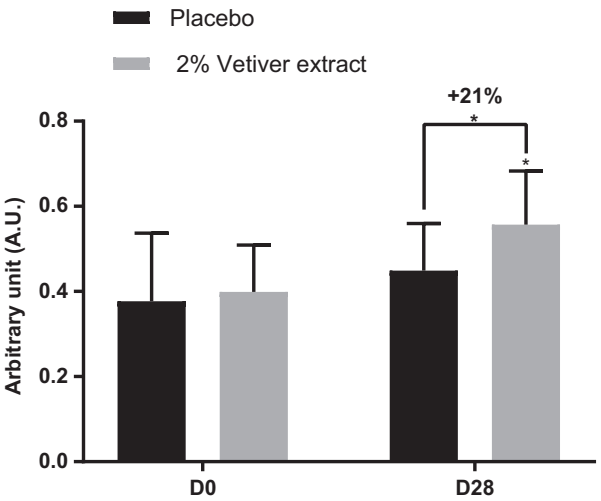

Figure 7 Impact of products containing or not containing Vetiver extract at 2% on lipid conformation after 28 days of application analysed by Raman spectroscopy. Results are presented as mean ± SD. Statistical analysis was performed using Kruskal–Wallis test followed by Mann–Whitney *U* test with * $P < 0.05$.

The results proved that the product containing Vetiver extract stimulates moisturizing of the skin and improves the lipid conformation of the skin at the clinical level after 28 days of application.

Improvement of quality and quantity of lipids from sebum

Secondly, we demonstrated that Vetiver extract was not only able to stimulate the synthesis of lipids from keratinocytes but also from sebocytes. The treatment of sebocytes cell line with Vetiver extract at 1% showed a significant induction of sebum production by +31% as observed by the lipid droplet formation from sebocytes after 7 days of treatment compared with the control (Figure 8). In overall skin lipids synthesis, we evidenced the production of some specific Free Fatty Acids (FFAs) from sebaceous cells model. The biochemical exploration was carried out on Lauric acid (C12:0)

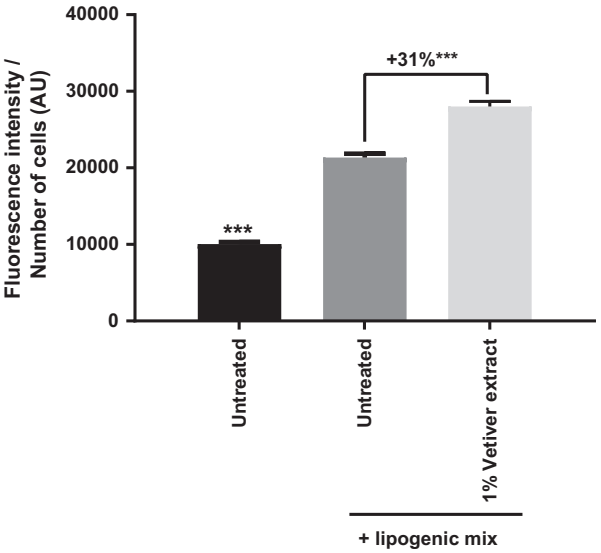

Figure 8 Lipid accumulation analysis in sebocytes cell line after 7 days of treatment with Vetiver extract at 1%. The results were compared with a stimulated control (untreated condition with lipogenic mix). Results of independent triplicate are presented as mean ± SEM. Statistical analysis was performed using ANOVA followed by Bonferroni's multiple comparisons test. The *P*-values presented are expressed in comparison with untreated condition with *** $P < 0.001$.

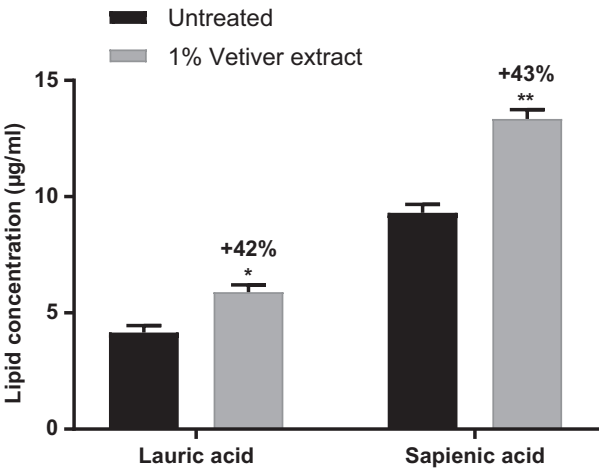

Figure 9 Lipid analysis and quantification after 7 days of treatment with Vetiver extract at 1%. Results of independent triplicate are presented as mean ± SEM. Statistical analysis was performed using ANOVA followed by Bonferroni's multiple comparisons test. The *P*-values presented are expressed in comparison with untreated condition with * $P < 0.05$ and ** $P < 0.01$.

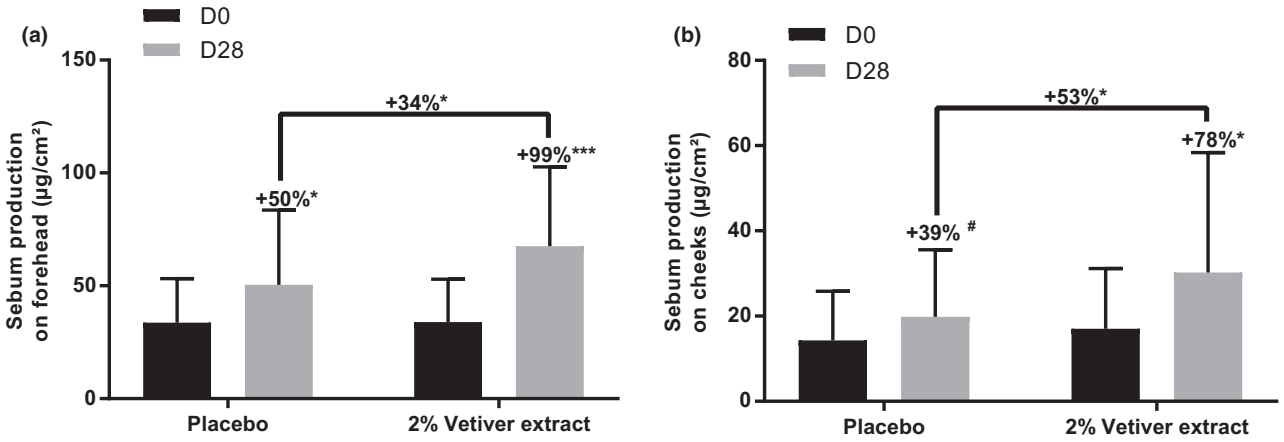

Figure 10 Panel a: Impact of products with or without Vetiver extract at 2% on forehead sebum production after 28 days of application. Panel b: Impact of products with or without Vetiver extract at 2% on cheek sebum production after 28 days of application. Results are presented as mean ± SEM. Statistical analysis was performed using Student's *t*-test in comparison with untreated condition with * $P < 0.05$, *** $P < 0.001$.

and Sapienic acid (C16:1, Δ6). After 7 days of treatment with Vetiver extract at 1% on sebocytes, the FFAs were collected and quantified by GC/MS. We showed that the extract induced a significant stimulation of the Lauric and Sapienic acids, by +42% and by +43%, respectively (Figure 9).

We performed a clinical study focusing on sebum production on mature panel members. More precisely, the study was carried out on 30 women aged 67 ± 2 years old who had a low facial sebum level. Volunteers twice daily applied the cream at 2% Vetiver extract or placebo cream in hemi face for 28 days. The sebum production was measured using Sebumeter® on two different areas including forehead and cheeks. We demonstrated that Vetiver extract at 2% induced a significant increase of sebum production on forehead and cheeks in comparison with D0 by +99% and +78% (Figure 10), respectively. Moreover, these inductions are significantly higher than the placebo with +34% on forehead and +53% on cheeks (Figure 10).

Therefore, we demonstrated that Vetiver extract at 2% is able to reactivate the sebum production that is drastically reduced on mature people.

Reshaping skin face by improvement of lipids synthesis from adipocytes

Thirdly, we evaluated the impact of Vetiver extract on the third source of skin lipids: the adipocytes. The lipogenesis activity has been evaluated on *ex vivo* full-skin explants via the measurement of the average of adipocyte size represented by the equivalent circular diameter (ECD). The distribution of adipocytes (in percentage) was ranged in three classes according to their size: the first part from 20 to 60 µm, the second from 60 to 100 µm and the third from 100 to more than 140 µm. For each class, the number of adipocytes was normalized according to the untreated values (Figure 11). In panel A, we can observe that the number of small adipocytes (class 20 to 60 µm) is significantly increased by 26% in presence of Vetiver extract. In the middle part of the range size (class 60 to 100 µm), there is a slight decrease in the number of adipocytes in panel B. Finally, in the panel C, we can observe that the extract significantly increased the number of large adipocytes (class 100 to 140 µm) by +189%. These results could be the consequence of two mechanisms; the increased number of the small adipocytes due to the recruitment of new adipocytes by adipogenesis process (adipocyte differentiation) and the increased number of the large adipocytes due to an increase of the volume of adipocytes (lipogenesis). Therefore, we then performed an evaluation of the ability of Vetiver extract to induce the adipogenesis on pre-adipocytes in order to confirm the hypothesis previously emitted. The pre-adipocytes were stimulated for 13 days with Vetiver extract at 3%. At the end of the treatment, the quantity of lipids was quantified via the mean fluorescence intensity of AdipoRed™ staining. We demonstrated that Vetiver extract was able to significantly increase the adipogenesis in pre-adipocytes by ×35 (Figure 12) and therefore induce the apparition of new small adipocytes.

The induction of lipogenesis on adipocytes can be reflected at *in vivo* level by a redefinition of volume due to the plumping effect that adipocytes can have on the tonicity, firmness and the fatigue of the skin. We carried out a clinical study to evaluate the skin fatigue parameter via the analysis of the R9 parameter. The evaluation was performed by Cutometer® on a panel of mature volunteers before and after 56 days of twice daily application of a cream containing or not the extract at 2%. After 56 days of application, we demonstrated a significant decrease by 23.2% of this parameter reflecting a significant improvement of skin biomechanical properties in comparison to D0 in presence of product containing the extract at 2%. It significantly decreased by 17.8% in comparison with placebo (Figure 13).

Finally, we analysed the anti-wrinkles efficacy of the product containing or not Vetiver extract at 2% after 56 days of application on skin face. The products were applied on the face on volunteers presenting wrinkles at the peri-labial area. After 56 days of application, we observed a significant reduction by 18.1% of wrinkles depth on peri-labial area on the volunteers treated with the product containing the extract in comparison to D0. Moreover, the comparison with placebo group evidenced a significant decrease of wrinkles depth by −4.3% with the product containing Vetiver extract at 2% even though the placebo showed a

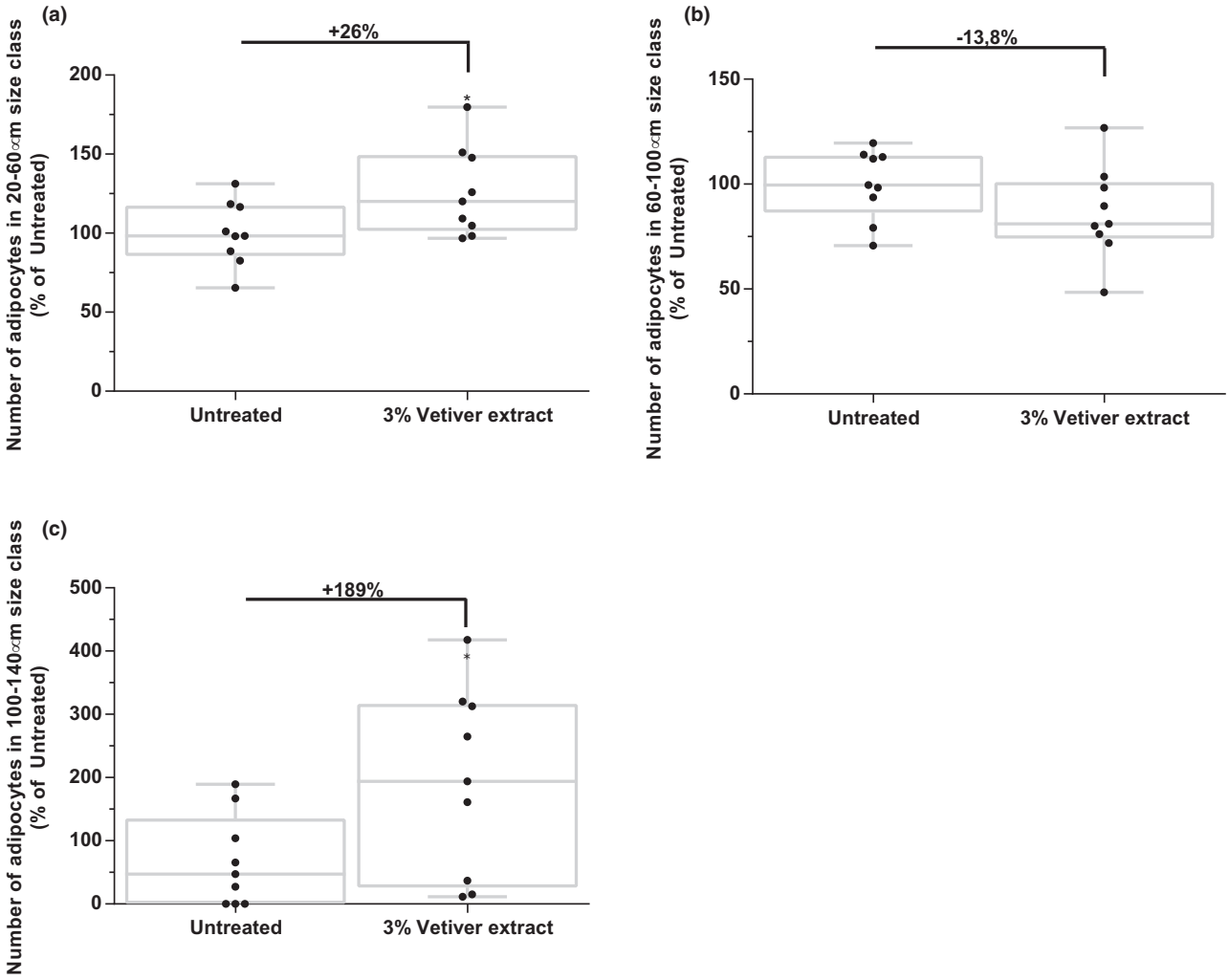

Figure 11 Impact of Vetiver extract at 3% on adipogenesis on *ex vivo* full skin. The number of adipocytes was ranged by ECD, in panel a from 20 to 60 μm, in panel b from 60 to 100 μm and in panel c from 100 to 140 μm. The results of independent triplicate are expressed in per cent of untreated condition as mean ± SEM. Statistical analysis was performed using Student's *t*-test in comparison with untreated condition with * $P < 0.05$ and *** $P < 0.001$.

significant decrease relative to D0 (−13.8%). This effect was observed on 100% of volunteers versus placebo (Figure 14).

Discussion

Skin integrity is a delicate balance, and its maintenance requires many factors to play a role. It is well described today that skin lipids have a major role in maintaining skin integrity [3,35]. As previously described, different lipids are produced by the skin cells, mainly by keratinocytes during the differentiation process, by sebocytes through sebum synthesis [36] and finally by adipocytes [21].

Intrinsic ageing disrupts this integrity by slowing the differentiation mechanism of keratinocytes and pre-adipocytes [16,30] and by reducing lipogenesis in sebocytes and adipocytes [26]. The renewal of skin lipids is disturbed and this results in dehydration, sagging of the skin and finally appearance of wrinkles [9,10,19].

We decided to evaluate the capability of Vetiver extract to boost the synthesis of skin lipids and to improve their quality. the traditional use of Vetiver essential oil was essentially based on its ability to act as anti-inflammatory, antimicrobial and healing agent but also as a potential moisturizing and rejuvenating agent (non-exhaustive properties) [1,2]. The dereplication of the Vetiver extract has identified zizanoic acid (ZA) as a major constituent which will serve as a chemical tracer for standardization. The extract has a specific composition that differentiates from Vetiver oil [34]. Haitian Vetiver oil is predominantly composed of primary alcohol sesquiterpenes khusimol and isovalencenol. Vetiver extract unicity comes from its richness in carboxylic acid sesquiterpenes: zizanoic acid and derivatives (compound Z and W), isovalencenic acid and compound Y; but also the presence of ketones, oplopanone, teuhetenone, solanerianone A. The composition being different from Vetiver oil, it was interesting to further peruse the biological evaluation of the extract.

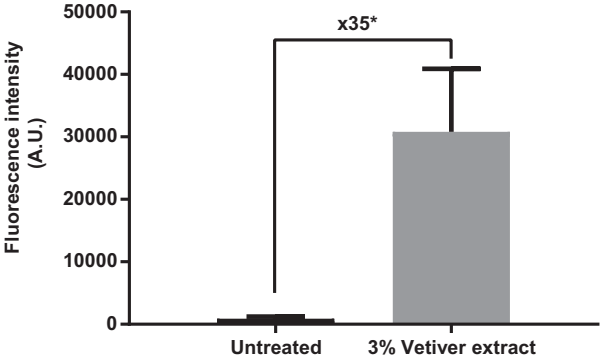

Figure 12 Impact of Vetiver extract at 3% on adipogenesis on pre-adipocytes. The results of independent triplicate are expressed in mean fluorescence intensity (A.U.) ± SEM. Statistical analysis was performed using Student's *t*-test in comparison with untreated condition with * $P < 0.05$.

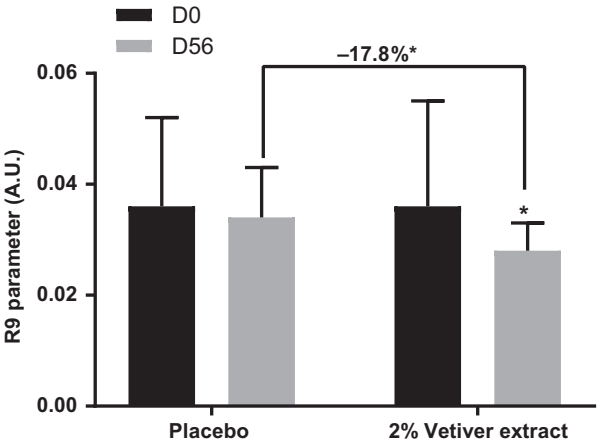

Figure 13 Impact of product with or without Vetiver extract at 2% on skin fatigue (R9 parameter) measured by Cutometer® after 56 days of application. Results are presented as mean ± SEM. Statistical analysis was performed using Student's *t*-test in comparison to Day 0 and with placebo group with * $P < 0.05$.

We firstly demonstrated that Vetiver extract was able to stimulate the lipid synthesis and their transport on keratinocytes. In the RHE model, we demonstrated that Vetiver extract induced the synthesis of sphingomyelin, phosphoglycerides, polar ceramides and cerebrosides. These lipids promote good integrity of the skin barrier [6,9]. Then we demonstrated using neutral lipid staining that, in the presence of Vetiver extract, the lipid levels on the skin dramatically increased. Continuing with this demonstration, in an *in vitro* culture of keratinocytes, we proved that Vetiver extract significantly stimulated the expression of CERT, a molecule involved in the transport of ceramides [5,7]. We also showed that Vetiver extract fortified the skin barrier by increasing the expression of involucrin and dermokine, two key proteins of the cornified layer [11]. This was in accordance to the keratinocyte morphology observed in our differentiation model. The overall improvement of the skin barrier related to this increase of lipid quantity was then confirmed by an *in vivo* study carried out on 20 volunteers using

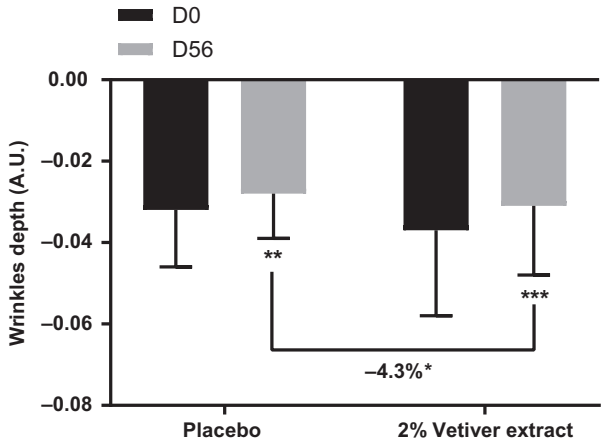

Figure 14 Wrinkles depth analysis from peri-labial area after 56 days of cream application containing or not Vetiver extract at 2% using AEVA-HE. Results are presented as mean ± SEM. Statistical analysis was performed using Student's *t*-test in comparison to Day 0 and with placebo group with * $P < 0.05$ and *** $P < 0.001$.

corneometry measurement. Skin hydration was significantly improved after 28 days of twice daily application of cream containing 2% of Vetiver extract. This increased hydration might be explained by the improvement of lipid conformation initiated by Vetiver extract. Indeed, thanks to the Raman spectroscopy analysis, the lipid conformation can be determined by measuring the ratio between the C-C *trans* and C-C *gauche* molecular bond. This ratio is associated with a compact state in the lipid packing [37], as observed with a treatment with Vetiver extract.

Skin integrity is also provided by the lipids from sebum. Indeed, these lipids are specific and contribute to the hydration of the skin [16]. Therefore, Vetiver extract was evaluated in an *in vitro* model of sebocytes where it significantly stimulated lipid synthesis after 7 days of treatment. These lipids also participate in the skin's immunity [15,17], so Vetiver extract was also evaluated with regards to its ability to improve the quality of the sebum via Antimicrobial Lipids (AML) production [17]. The presence of Vetiver extract increased the content of Lauric acid and Sapienic acid, two recognized AMLs. Finally, we confirmed the impact of Vetiver extract on sebum production on 30 mature women who had very low levels of facial sebum. A low level of sebum is known to be correlated to skin dryness [26]. After 28 days of application of the cream at 2% of Vetiver extract, we demonstrated a clear and significant improvement of the quantity of sebum on the volunteers' cheeks and foreheads.

Boosting the adiposity was the final step on our global anti-ageing strategy; the facial fat loss of adipose tissue having a direct impact on the appearance of wrinkles [19,28]. We decided to evaluate the ability of Vetiver extract to stimulate the adipogenesis of pre-adipocytes and lipogenesis of adipocytes. We demonstrated on *ex vivo* skin full explants that the extract was able to significantly increase the number of small adipocytes, supposing an impact on adipogenesis. The stimulation of pre-adipocytes differentiation was then confirmed at *in vitro* level. Moreover, Vetiver extract exerted an activity on adipocytes maturation on the skin explants, demonstrated by the increase of large adipocytes number. The hypothesis of filling the wrinkles by boosting the adipocytes maturation and

their number was then confirmed at the clinical level. We studied the anti-wrinkles efficacy of Vetiver extract on a large panel of 42 mature volunteers who had fine to deep wrinkles on the studied areas in order to demonstrate its effect in all types of wrinkles. The biometric measures showed an improvement of skin fatigue. The wrinkles depths from peri-labial area after 56 days were decreased significantly in both groups. The effect of the placebo formula is not negligible but the improvement brought by the extract was significantly higher in comparison with the placebo. This study allowed us to confirm a clear anti-ageing effect of the extract.

Conclusion

Nowadays, Vetiver roots are mainly used in the perfume industry as an iconic plant and become a waste once they are distilled. We started with the idea that the upcycling of exhausted Vetiver roots could bring with it a new trend of consumption in the cosmetic industry.

We demonstrated both *in vitro* and *ex vivo* that the extract was able to increase the quantity and the quality of lipids produced by keratinocytes, sebocytes and adipocytes leading to an overall renewal of skin lipids. Then, via various clinical studies, we confirmed in a panel of mature women the efficacy of the extract on increasing skin hydration, sebum content and decreasing of ageing signs.

We thus conclude that Vetiver extract has an anti-ageing effect by promoting the renewal of lipids in each skin layer.

Acknowledgements

The authors wish to thank Dr Sandre's team from Polyclinique Courlancy (Reims, France) for their support in providing skin biopsies. The authors would like also to thank their partners BioAlternatives (Gençay, France), Bio-EC (Longjumeau, France), Synelvia (Labège, France) and M Conseils (Reims, France) for their help in conducting these studies.

References

1. Balasankar D, Vanilarasu K, Preetha PS, Umadevi SRM, Bhowmik D. Senna-a medical miracle plant. *J Med Plants Studies*. 2013;**2**(3):11.
2. Chomchalow N. *Other Uses and Utilization of Vetiver*. 12.
3. Elias PM. Stratum corneum defensive functions: an integrated view. *J Invest Dermatol*. 2005;**125**(2):183–200.
4. Nishifuji K, Yoon JS. The stratum corneum: the rampart of the mammalian body: Stratum corneum in mammals. *Vet Dermatol*. 2013;**24**(1):60–e16.
5. Rabionet M, Gorgas K, Sandhoff R. Ceramide synthesis in the epidermis. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids*. 2014;**1841**(3):422–434.
6. van Smeden J, Janssens M, Gooris GS, Bouwstra JA. The important role of stratum corneum lipids for the cutaneous barrier function. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids*. 2014;**1841**(3):295–313.
7. Hanada K. Intracellular trafficking of ceramide by ceramide transfer protein. *Proc Jpn Acad Ser B*. 2010;**86**(4):426–437.
8. Hanada K, Kumagai K, Tomishige N, Yamaji T. CERT-mediated trafficking of ceramide. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids*. 2009;**1791**(7):684–691.
9. Rawlings AV, Matts PJ. Stratum corneum moisturization at the molecular level: an update in relation to the dry skin cycle. *J Invest Dermatol*. 2005;**124**(6):1099–1110.
10. Krutmann J, Bouloc A, Sore G, Bernard BA, Passeron T. The skin aging exposome. *J Dermatol Sci*. 2017;**85**(3):152–161.
11. Leclerc EA, Huchenq A, Kezic S, Serre G, Jonca N. Mice deficient for the epidermal dermokine and isoforms display transient cornification defects. *J Cell Sci*. 2014;**127**(13):2862–2872.
12. Raymond A-A, de Peredo AG, Stella A, Ishida-Yamamoto A, Bouyssie D, Serre G, *et al*. Lamellar bodies of human epidermis: proteomics characterization by high throughput mass spectrometry and possible involvement of CLIP-170 in their trafficking/secretion. *Mol Cell Proteom*. 2008;**7**(11):2151–2175.
13. Toulza E, Galliano M-F, Jonca N, Gallinaro H, Méchin M-C, Ishida-Yamamoto A, *et al*. The human dermokine gene: description of novel isoforms with different tissue-specific expression and subcellular location. *J Invest Dermatol*. 2006;**126**(2):503–506.
14. Zouboulis CC, Baron JM, Böhm M, Kippenberger S, Kurzen H, Reichrath J, *et al*. Frontiers in sebaceous gland biology and pathology. *Exp Dermatol*. 2008;**17**(6):542–551.
15. De Luca C, Valacchi G. Surface lipids as multifunctional mediators of skin responses to environmental stimuli. *Mediators Inflamm*. 2010;**2010**:1–11.
16. Verdier-Sévrain S, Bonté F. Skin hydration: a review on its molecular mechanisms. *J Cosmet Dermatol*. 2007;**6**(2):75–82.
17. Fischer CL, Blanchette DR, Brogden KA, Dawson DV, Drake DR, Hill JR, *et al*. The roles of cutaneous lipids in host defense. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids*. 2014;**1841**(3):319–322.
18. Lovászi M, Szegedi A, Zouboulis CC, Törőcsik D. Sebaceous-immunobiology is orchestrated by sebum lipids. *Dermatoendocrinol*. 2017;**9**(1):e1375636.
19. Bojanowski K. Hypodermal delivery of cosmetic actives for improved facial skin morphology and functionality. *Int J Cosmet Sci*. 2013;**35**(6):562–567.
20. Tchkonia T, Morbeck DE, Von Zglinicki T, Van Deursen J, Lustgarten J, Scrable H, *et al*. Fat tissue, aging, and cellular senescence: Fat tissue and aging. *Aging Cell*. 2010;**9**(5):667–684.
21. Luo L, Liu M. Adipose tissue in control of metabolism. *J Endocrinol*. 2016;**231**(3):R77–99.
22. Rivera-Gonzalez G, Shook B, Horsley V. Adipocytes in skin health and disease. *Cold Spring Harb Perspect Med*. 2014;**4**(3):a015271.
23. Wasko RR, Horsley V. Thin skinned: aged adipocyte atrophy impacts innate immunity. *Trends Immunol*. 2019;**40**(3):175–177.
24. Zhang L, Chen SX, Guerrero-Juarez CF, Li F, Tong Y, Liang Y, *et al*. Age-related loss of innate immune antimicrobial function of dermal fat is mediated by transforming growth factor beta. *Immunity*. 2019;**50**(1):121–136.e5.
25. Ishikawa J, Yoshida H, Ito S, Naoe A, Fujimura T, Kitahara T, *et al*. Dry skin in the winter is related to the ceramide profile in the stratum corneum and can be improved by treatment with a *Eucalyptus* extract. *J Cosmet Dermatol*. 2013;**12**(1):3–11.
26. Downing DT, Wertz PW, Stewart ME. The role of sebum and epidermal lipids in the cosmetic properties of skin. *Int J Cosmet Sci*. 1986;**8**(3):115–123.
27. Elias PM, Schmuth M. Abnormal skin barrier in the etiopathogenesis of atopic dermatitis. *Curr Opin Allergy Clin Immunol*. 2009;**9**(5):437–446.
28. Chon S-H, Pappas A. Differentiation and characterization of human facial subcutaneous adipocytes. *Adipocyte*. 2015;**4**(1):13–21.

29. Cartwright M, Tchkonia T, Kirkland J. Aging in adipocytes: Potential impact of inherent, depot-specific mechanisms. *Exp Gerontol.* 2007;**42**(6):463–471.
30. Kirkland JL, Dobson DE. Preadipocyte function and aging: links between age-related changes in cell dynamics and altered fat tissue function. *J Am Geriatr Soc.* 1997;**45**(8):959–967.
31. Brett E, Chung N, Leavitt WT, Momeni A, Longaker MT, Wan DC. A review of cell-based strategies for soft tissue reconstruction. *Tissue Eng Part B Rev.* 2017;**23**(4):336–346.
32. Hubert J, Nuzillard J-M, Purson S, Hamzaoui M, Borie N, Reynaud R, *et al*. Identification of natural metabolites in mixture: a pattern recognition strategy based on 13 C NMR. *Anal Chem*. 2014;**86**(6):2955–2962.
33. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 1959;**37**(8):911–917.
34. Filippi J-J, Belhassen E, Baldovini N, Brevard H, Meierhenrich UJ. Qualitative and quantitative analysis of vetiver essential oils by comprehensive two-dimensional gas chromatography and comprehensive two-dimensional gas chromatography/mass spectrometry. *J Chromatogr A.* 2013;**1288**:127–148.
35. Feingold KR, Elias PM. Role of lipids in the formation and maintenance of the cutaneous permeability barrier. *Biochim Biophys Acta BBA - Mol Cell Biol Lipids.* 2014;**1841**(3):280–294.
36. Pappas A. Epidermal surface lipids. *Dermatoendocrinol.* 2009;**1**(2):72–76.
37. Choe C, Lademann J, Darvin ME. A depth-dependent profile of the lipid conformation and lateral packing order of the stratum corneum in vivo measured using Raman microscopy. *Analyst*. 2016;**141**(6):1981–1987.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Description of the lipid standards used in TLC for the analysis of polar lipids.

Data S1. Chemical structures and experimental ^{1}H and ^{13}C NMR chemical shifts values of the major metabolites identified in the *Chrysopogon zizanioides* exhausted root extract.