

## ORIGINAL ARTICLE

# In vitro, ex vivo and clinical approaches to evaluate the potential effect of *Gentiana lutea* extract on skin

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## Abstract

**Background:** Dark circles affect subjects of all ages and in all skin types. They can be treated by various methods, particular by topical solutions. This investigation was directed towards exploring the effect of gentiopicroside (GP) on the skin around the eyes. For this, an extract of *Gentiana lutea* (GIE) containing GP (65% by dry matter) was evaluated on oxidant and angiogenesis parameters using in vitro and ex-vivo studies. A clinical experimentation was also realized.

**Methods:** The effect of GIE at different concentrations on antioxidant gene was evaluated in vitro by RT-qPCR after treatment of NHDF. The effect of 2.93  $\mu\text{g mL}^{-1}$  GIE on the release of VEGF-A and VEGF-C by NHDF was also studied. The effect of 87.9  $\mu\text{g mL}^{-1}$  GIE was also evaluated on pseudotube formation in a coculture system of normal dermal microvascular endothelial cells (HMVEC-d)-NHDF stimulated or not with VEGF as pro-angiogenic factor. Prior to these assays, preliminary cytotoxicity assays were performed using a standard WST-8 reduction assay. The expressions of carboxymethyl-lysine and glyoxalase-1 were quantified on skin explants topically treated with 147  $\mu\text{g mL}^{-1}$  GIE in basal and UVA-irradiated conditions. A clinical study was conducted in 22 subjects using topical twice daily for 14 days on eye area (split-face application: cream containing 147  $\mu\text{g mL}^{-1}$  GIE versus placebo). 3D image acquisition and skin colour measurement were performed at D0 and D14.

**Results:** Treatment of GIE upregulated the gene expression of NFE2L2 and downregulated the expression of CXCL8. GIE targeted AGEs pathways and reduced the formation of pseudotubes. A total of 147  $\mu\text{g mL}^{-1}$  GIE gel cream significantly reduced significantly the average roughness and relief of the upper eyelid skin as well as the redness of dark circles after 14 days of application.

**Conclusion:** By acting on the pathway of AGEs, VEGF-A and VEGF-C, GIE seems to allow a rejuvenation of the skin resulting, among others, in a decrease in redness. It now would be interesting to evaluate the efficacy of GIE on skin around eyes microbiota, antibacterial gentiopicroside property being well-established.

## KEYWORDS

advanced glycation endproducts, gentiopicroside, skin physiology/structure

## Résumé

**Contexte:** Le contour des yeux est une zone sensible. Les cernes affectent les sujets de tout âge et de tout type de peau. Différentes solutions peuvent être proposées, dont les solutions topiques. Cette étude visait à explorer l'effet d'un extrait de *Gentiana lutea* (GIE) riche en gentiopicroside (65% de matière sèche) sur des paramètres d'oxydation et d'angiogenèse au moyen d'études *in vitro* et *ex-vivo*. Une expérimentation clinique a également été réalisée.

**Méthodes:** L'effet du GIE à différentes concentrations sur des gènes antioxydants a été évalué *in vitro* par RT-qPCR après traitement de fibroblastes (NHDF). L'effet de 2.93 µg mL<sup>-1</sup> GIE sur la libération de VEGF-A et VEGF-C a également été étudié. Il en est de même pour l'effet de 87.9 µg g mL<sup>-1</sup> GIE sur la formation de pseudotubes qui a été évalué dans un système de co-culture de cellules endothéliales (HMVEC-d)- NHDF stimulées ou non avec du VEGF comme facteur pro-angiogénique. Les expressions de la carboxyméthyl-lysine et de la glyoxalase-1 ont été quantifiées sur des explants cutanés traités par voie topique avec 147 µg g mL<sup>-1</sup> GIE dans des conditions basales et irradiées par UVA. Une étude clinique a été menée sur vingt-deux sujets en utilisant un traitement topique deux fois par jour pendant 14 jours sur le contour des yeux (crème contenant 147 µg g mL<sup>-1</sup> GIE contre placebo). L'acquisition d'images 3D et la mesure de la couleur de la peau ont été réalisées à J0 et J14.

**Résultats:** Le traitement par GIE a augmenté l'expression génétique de NFE2L2 et diminue l'expression de CXCL8. GIE a ciblé les voies des AGEs et a réduit la formation de pseudotubes. 147 µg g mL<sup>-1</sup> GIE gel crème a significativement réduit la rugosité moyenne et le relief de la peau de la paupière supérieure ainsi que la rougeur des cernes après 14 jours d'application.

**Conclusion:** En agissant sur la voie des AGEs, du VEGF-A et du VEGF-C, GIE semble permettre un rajeunissement de la peau se traduisant, entre autres, par une diminution des rougeurs. Il serait maintenant intéressant d'évaluer l'efficacité du GIE sur le microbiote de la peau du contour des yeux, la propriété antibactérienne du gentiopicroside étant bien établie.

## INTRODUCTION

Dark circles, a common condition used to express the relative darkness of the skin around the eyes, affect subjects of all ages and in all skin types [1]. They can affect the quality of life and appearance, as they can make sufferers look tired. They can be caused by a variety of conditions such as infection, inflammation, allergies and lifestyle factors (e.g. insufficient sleep, smoking and mental stress). Other major contributing factors include soft tissue, blood flow stagnation and ageing [2, 3]. Even if the physiopathology of skin ageing is multifactorial, cell degeneration involving the accumulation of advanced glycation end-products (AGEs) is a key factor [4]. Indeed, production of AGEs on collagen leads to cross linking inducing abnormalities in the extracellular matrix, impairing cell-matrix interaction

and leading to skin ageing. AGEs also bind to specific receptors on immune cells, which trigger the release of inflammatory mediators and generation of reactive oxygen species (ROS) leading to increase in the production of AGEs damage. Among these AGEs are the glycoxidation products, N<sup>ε</sup>-(carboxymethyl)lysine (CML) and pentosidine, which are the only chemically characterized AGEs known to accumulate in protein with age [5]. AGEs also modulate the function of endothelial cells affecting the expression of angiogenic factors by increasing vascular endothelial growth factor-A (VEGF-A), inducing an increase in capillary permeability [6]. The detoxification of AGEs can be realized through the glyoxalase system. Among this system, Glyoxalase 1 (Glo-1) catalyses the primary detoxification step, alteration of Glo-1 protein being involved in many pathological processes in ageing. It is

also important to note that Glo-1 expression is positively regulated by Nrf2. Ageing also results in gradual atrophy and decrease in density and network complexity of the lymphatic system, promoting water accumulation in tissues [7], VEGF-C promoting the functional lymphatic vessels in adults [8]. Its function is to drain the protein-rich lymph from the extracellular space, and to mediate the immune response, particularly in inflammatory conditions by draining extravasated fluid, antigens and inflammatory mediators [7]. Dark circles can be treated by various methods, such as normobaric oxygen therapy, depigmenting agents, chemical peeling, lasers and hyaluronic fillers [2, 8]. Besides these treatments, topical solutions including caffeine or retinol can be proposed. It also seems that gentiopicroside (GP), an anti-ageing secoiridoid glycoside [9], known for its wide range of pharmacological activities and obtained from plants belonging to gentian species that are used extensively as medicinal herbs, can be a good candidate for reducing skin concerns in the eye area. Indeed, GP has anti-inflammatory, antioxidant, antibacterial activities and smooth muscle relaxing effect [10, 11]. GP has also been documented to reduce tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) concentrations and VEGF-A expression [12].

Thus, this investigation was directed towards exploring the protective effect of GP on the skin around the eyes. For this, an extract of *Gentiana lutea* (GIE) containing gentiopicroside (65% of dry matter) was evaluated on oxidant and angiogenesis parameters using in vitro and ex vivo studies. A clinical experimentation was also realized, focusing on the roughness of the relief and the redness.

## MATERIALS AND METHODS

### GIE extract

GIE extract was realized using hydro-alcoholic extraction. The intermediate extract was concentrated thanks to alcohol evaporation and then purified by chromatography to collect a fraction which is highly purified in gentiopicroside. The latter was finally stabilized in water/propanediol. The extract also contains polyphenols (around 3% of dry matter), polysaccharides (around 3% of dry matter) and sugars (around 8% of dry matter).

### In vitro evaluation

#### Mitochondrial activity

Mitochondrial activity of normal human dermal fibroblasts (NHDF) and human dermal microvascular

endothelial cells (HMVEC-d) after different percentage of GIE treatment as five concentrations (2.93, 8.79, 29.3, 87.9 and 293  $\mu\text{g mL}^{-1}$ ) were assessed through the enzymatic conversion of tetrazolium salt (WST-8 assay kit; Sigma Aldrich), and was used as a parameter to monitor cell survival and therefore cytotoxicity. After 72 h of treatment, NHDF or HMVEC were incubated during 7 days with WST-8 reduced in a water-soluble orange coloured product (formazan) by succinate dehydrogenase (mitochondrial enzyme). This transformation is proportional to the number of living cells and their metabolic activity. The optical density (OD) of the extracts at 450 nm was recorded with a spectrometer (VERSAmax; Molecular Devices). In addition, the cells were observed under the microscope.

### Effect on GIE on gene expression

NHDF were cultured at a concentration of 120 000 cells per well in a 24-well plate. After adhesion (24 h after seeding), the cells were rinsed with HBSS and then treated with the GIE diluted at different concentrations in the culture medium without supplements and without phenol red for 24 h. Each condition was carried out in culture triplicate. The cell layers of the triplicates were pooled. Then, the mRNAs were extracted, and reverse transcription was performed. Real-time PCR (qPCR) was performed using cDNA obtained from 20 ng of mRNA in a 384-well plate in a final volume of 10  $\mu\text{L}$  according to the manufacturer's recommendations. Each amplification point was performed in triplicate. Each  $C_T$  obtained was then brought back to the reference gene HPRT1 to calculate the  $\Delta C_T$  and the normalized expression is calculated using the formula  $2^{-\Delta C_T}$ . The results were expressed as percentage of overexpression or underexpression relative to the reference condition. Since triplicates from each cell condition were pooled prior to mRNA extraction, the results represent the mean of the three wells.

### Effect of GIE extract on VEGF-A and VEGF-C protein released by NHDF

NHDF were seeded in 96-well plates and cultured for 24 h in culture medium. The medium was then replaced by culture medium containing or not 2.93  $\mu\text{g mL}^{-1}$  GIE, and the inflammatory inducer, Phorbol 12-Myristate 13-Acetate (PMA) at 5  $\mu\text{g mL}^{-1}$ . Then, the cells were incubated for 48 h. All experimental conditions were performed in  $n=3$ . At the end of incubation, the culture supernatants were collected for VEGF quantification. The quantity of

VEGF-A and VEGF-C released in culture supernatants was measured using ELISA kits according to the supplier's instructions.

## Effect of GIE on pseudotube formation

### Basal conditions

HMVEC/NHDF co-culture (1:2 ratio) was seeded in a 96-well plate and cultured for 24 h. The medium was then removed and replaced by co-culture medium containing or not (control),  $87.9 \mu\text{g mL}^{-1}$  GIE or VEGF, tested at  $100 \text{ ng mL}^{-1}$  and the cells were incubated for 7 days with a treatment renewal after 72 h of incubation.

### Stimulated conditions

The HMVEC/NHDF co-culture (1:2 ratio) was seeded in a 96-well plate and cultured for 24 h. The medium was then removed and replaced by co-culture medium containing or not (stimulated control),  $87.9 \mu\text{g mL}^{-1}$  GIE or the reference (suramin at  $100 \mu\text{M}$ ) and VEGF at  $100 \text{ ng mL}^{-1}$  was added. The cells were incubated for 7 days with treatment renewal after 72 h of incubation. In parallel, a non-stimulated control was performed. All experimental conditions were performed in  $n=3$ . After incubation, the co-culture medium was discarded, and the cells were fixed and permeabilized. The cells were then labelled using an anti-vWF (von Willebrand factor) primary antibody. The primary antibody was then revealed using an appropriate fluorescent secondary antibody (GAR-Alexa 488) and the cell nuclei were stained in parallel using Hoechst 33258 solution (bis-benzimide). The formation of pseudotubes was observed using a NIKON Diaphot 300 microscope (objective lens  $\times 4$ ). The images were captured (one photo per well) using a NIKON DS-Fi1 camera and NIS-Elements 4.13.04 software. The labelling was quantified by measuring the total area of the pseudotubes using ImageJ software. The analysis of pseudotube formation was performed through vWF labelling.

Note: Before performing the image segmentation for the quantification of the total area of pseudotubes, the contrast of all images was enhanced to allow the optimal detection of pseudotubes and all images were analysed using the same settings.

## Ex vivo evaluation

### Effect of GIE on advanced glycation end products

The protective efficacy of  $147 \mu\text{g mL}^{-1}$  GIE was evaluated on an ex vivo model of human skin exposed (or not) to UVA irradiation through:

1. in situ analysis of CML ( $\text{N}^{\epsilon}$ -(carboxymethyl)lysine; one of the major products of advanced glycation [AGE]) levels on proteins
2. the analysis of the protein expression of Glyoxalase-1 (detoxification enzyme of glycation products).

Twelve explants of approximately 10 mm ( $\pm 1$  mm) in diameter were prepared from a tummy tuck of a 42-year-old, phototype III, female donor (reference BIOP-2021-03-30-2). The explants were put into survival at D0 in 2 mL of DMEM medium (high glucose  $4.5 \text{ g L}^{-1}$ ; glutaMAX) supplemented with SVF (fetal calf serum, 10%), penicillin-streptomycin (1%), at  $37^{\circ}\text{C}$  in a humid atmosphere, enriched with 5%  $\text{CO}_2$ . The culture medium was renewed every 24 h. Explants were divided into batches and each experimental batch consisted of three replicates ( $n=3$ ). Solutions (solvent propanediol and  $147 \mu\text{g mL}^{-1}$  GIE) were prepared in aqueous solutions. The solutions were filtered (on  $0.22 \mu\text{m}$  pore size filter) before topical application to explants. On day D0,  $30 \mu\text{L}$  of the solutions were applied, topically to the explants, according to the experimental conditions as follows: (1) treatment with solvent, non-irradiated, (2) treatment with GIE non-irradiated (3) treatment with solvent, irradiated-stress and (4) treatment with GIE, irradiated-stress. On day D1, explants from the 'solvent+ stress' and 'GIE + stress' batches were exposed to UVA (two cycles of irradiation, with a cumulative dose of  $6 \text{ J cm}^{-2}$ ; 365 nm LED source). Immediately after irradiation, the explants were returned to  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for 3 h, and the culture medium was renewed. Three hours after the irradiation cycle, explants from each batch were harvested, embedded in OCT for cryopreservation and stored at  $-80^{\circ}\text{C}$  until analysis.

### Detection, visualization and quantification of biomarkers (CML; Glyoxalase-1)

Explant sections of  $4 \mu\text{m}$  thickness were obtained in the Cryostat (Leica) and fixed on a microscope slide. A saturation step of nonspecific sites was performed with a 5% solution of BSA (bovine serum albumin, for Glyoxalase-1) or protein-free blocking buffer (Pierce<sup>TM</sup>, for CML) in PBS (phosphate buffered saline, pH 7.4). Next, explant sections were independently incubated with the primary antibody specific for the biomarker of interest in 5% BSA or Pierce<sup>TM</sup> buffer in PBS. After rinsing the primary antibody with 5% BSA or Pierce<sup>TM</sup> buffer in PBS, the sections were incubated with the fluorochrome-coupled secondary antibody (Alexa Fluor 647). Nucleus labelling was performed with DAPI (4',6-diamidino-2-phenylindole). Finally, excess antibody and DAPI were removed with a sequence of rinses with PBS. Each set of images (one per

biomarker) was acquired with an epifluorescence microscope (ThermoFisher, Evos M5000) using strictly the same acquisition time and resolution (40× and 10× objective).

Images collected and saved with all fluorescence signal intensity levels were analysed using ImageJ software (Rasband, NIH). For each biomarker, quantification was performed on each image by integration of the specific fluorescence signal normalized by the area of the evaluation area. Three images per condition were analysed to obtain a mean value and standard deviation. Finally, these values were normalized with respect to the vehicle-treated and non-irradiated group (considered at 100%). Statistical analyses were performed using the 'GraphPad' software.

## Quantification

Three images per experimental group were used for quantification of CML levels by integrating the specific fluorescence signal normalized by the area of evaluation.

The same methodology was used for the expression of Glyoxalase-1.

## Clinical evaluation

This single centre randomized double-blinded interventional split-face study controlled clinical trial adhered to the principles of Good Clinical Practices and the declaration of Helsinki. According to local and European regulatory guidelines (Official Journal of EU of 10 March 2010 paragraph 1.2.9), this type of trial testing marketed cosmetics do not require approval from local ethics committees.

Both sides of the face were treated with the same amount of gel cream. The major difference in the treatment of both facial sides was that one side of the face was treated with placebo, whereas the contralateral side was treated with 147  $\mu\text{g mL}^{-1}$  GIE formulated in a gel cream.

Participants were informed about the goals and the methodology of this study and informed consent and photography consent were obtained from each subject before any study-related procedure took place.

## Subjects

The main inclusion criteria were 22 Caucasian subjects (French origin) aged between 43 and 64 years. All healthy subjects had (1) saggy upper eyelids and dark circles (2) all had normal to combination skin and (3) were classified with a phototype of I to III on the Fitzpatrick scale. Both formulations were tested in 22 subjects. They were encouraged to refrain from any topical application on their face,

apart from the test products, throughout the whole study duration. Assessments of irritant, phototoxic potential and skin compatibility of the gel creams were realized before its application, as well as the eye irritation (HET-CAM). The agent is tolerated as a cosmetic by the European authorities.

## Test formulations

The gel cream containing 147  $\mu\text{g mL}^{-1}$  GIE and placebo had the following components:

Aqua, glycerine (ammonium acryloyldimethyl taurate/VP copolymer, ammonium acryloyldimethyl taurate/beheneth-25 methacrylate cross polymer and isopropyl miristate pentylene glycol). In addition to these, the «active» gel cream also contains 147  $\mu\text{g mL}^{-1}$  GIE.

Both study formulas were identical in appearance and supplied with two identical pump dispensers.

## Treatment and evaluation visits

The gel cream or the placebo (split-face) was applied twice daily to the eye contour for 14 days (each dose applied was 0.4 g). The subjects were evaluated on Day 0 (D0) and D14. The maximum study duration was 14 days for all subjects. Efficacy end points were realized through a 3D image acquisition using DermaTop<sup>®</sup> and skin colour measurement (Spectrophotometer<sup>®</sup>). DermaTop<sup>®</sup> is contactless system based on the use of fringe projection. The system calculates a phase image from images with interference fringe projection. This image then allows to determine the height of each point. It permits the evaluation of the upper eyelid skin ageing and the severity of the tear trough (roughness, volume and mean depth). CM700-d Spectrophotometer<sup>®</sup> (MINOLTA) permits to evaluate the colour of dark circles. It converts colours perceived by man to a digital code composed of three parameters:  $L^*$ : for clarity;  $a^*$ : for to green-to-red spectrum; and  $b^*$ : for the blue-to-yellow spectrum. In the evaluation of the dark circle colour, a decrease in  $a^*$  parameter characterizes an attenuation of the red pigmentation of dark circles. Dermatological and ophthalmological evaluations were conducted at D14.

## Statistical analysis

Data are presented as mean  $\pm$  standard error of mean (SEM). The normality of data distribution was evaluated using the Shapiro–Wilk test. Data have been analysed by Wilcoxon signed-rank test or Student's *t*-test (according to the distribution) with GraphPad Prism 7 (GraphPad Software). The split-face design provides a consistent

baseline allowing repeated measurements and analysis, enabling each subject to be his own control. The significance level was set at 5%.

## RESULTS

### In vitro data

#### Cytotoxicity assessment

No effect of GIE treatment, whatever the concentration used, was noted on the viability of NHDF and HMVEC-d (Figure 1).

#### Gene expression analysis

Treatment of GIE up-regulated the gene expression of nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2*) as follows:

- +38% at  $87.9 \mu\text{g mL}^{-1}$  GIE, +30% at  $29.3 \mu\text{g mL}^{-1}$  GIE, +21% at  $8.79 \mu\text{g mL}^{-1}$  GIE and +14% at  $2.93 \mu\text{g mL}^{-1}$  GIE for *NFE2L2*.
- 8% at  $29.3 \mu\text{g mL}^{-1}$  GIE, -22% at  $8.79 \mu\text{g mL}^{-1}$  GIE and -21% at  $2.93 \mu\text{g mL}^{-1}$  GIE for *CXCL8*.

#### Effect of GIE extract on VEGF-A and VEGF-C protein released by NHDF

##### VEGF-A

A low level of VEGF-A was secreted by NHDF ( $86 \text{ pg mL}^{-1}$ ) and the stimulation of NHDF with  $5 \mu\text{g mL}^{-1}$  PMA

induced a release of VEGF-A ( $131 \text{ pg mL}^{-1}$ ). These results were expected and validated the assay. A total quantity of  $2.93 \mu\text{g mL}^{-1}$  GIE induced a decreased release of VEGF-A by NHDF (-15%,  $p < 0.01$  vs. control, Figure 2).

##### VEGF-C

It was secreted by NHDF ( $1535 \text{ pg mL}^{-1}$ ) and the stimulation of NHDF with  $5 \mu\text{g mL}^{-1}$  PMA stimulated its release ( $2943 \text{ pg mL}^{-1}$ ). A total quantity of  $2.93 \mu\text{g mL}^{-1}$  GIE induced a concentration stimulating effect on VEGF-C release (+13% vs. control,  $p < 0.05$ ).

#### Effect of GIE on pseudotube formation

The effect of GIE was evaluated on pseudotube formation in a co-culture model of HMVEC-d cells with NHDF stimulated or not with VEGF as this pro-angiogenic factor induces the organization of endothelial cells into pseudotubes after several days of incubation. Quantification of pseudotubes formation in co-culture of NHDF-HMVEC-d, after GIE treatment in both basal (A) and VEGF-stimulated (B) conditions are presented in Figure 3a. In the non-stimulated control condition, the immunolabelling observed was weak, therefore indicating the absence of organization of endothelial cells under basal condition. The treatment of the co-culture with  $100 \text{ ng mL}^{-1}$  VEGF for 7 days resulted in the organization of endothelial cells into pseudotubes and the reference suramin, tested at  $100 \mu\text{M}$ , strongly inhibited the VEGF-induced pseudotube formation (-89%). These results were expected and validated the assay.

Under VEGF-stimulated conditions,  $87.9 \mu\text{g mL}^{-1}$  GIE induced a significant inhibition of VEGF-induced pseudotube formation in the HMVEC/NHDF co-culture (-33%,  $p < 0.05$ ) (Figure 3b).

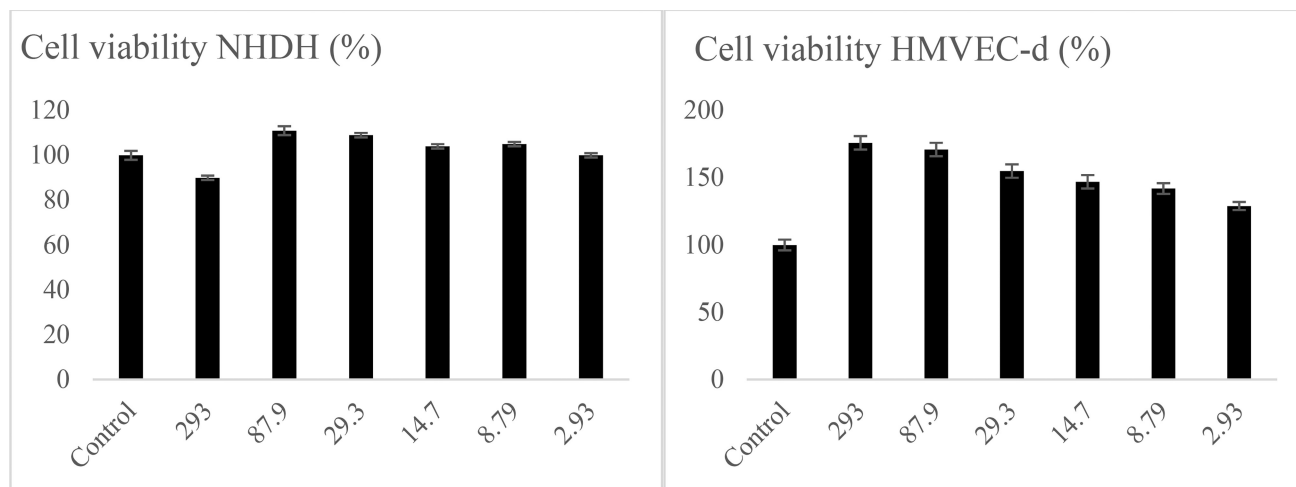
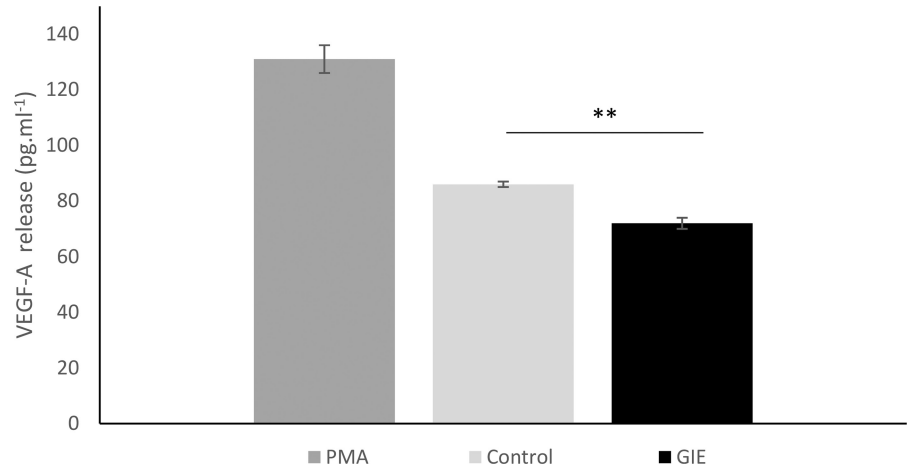


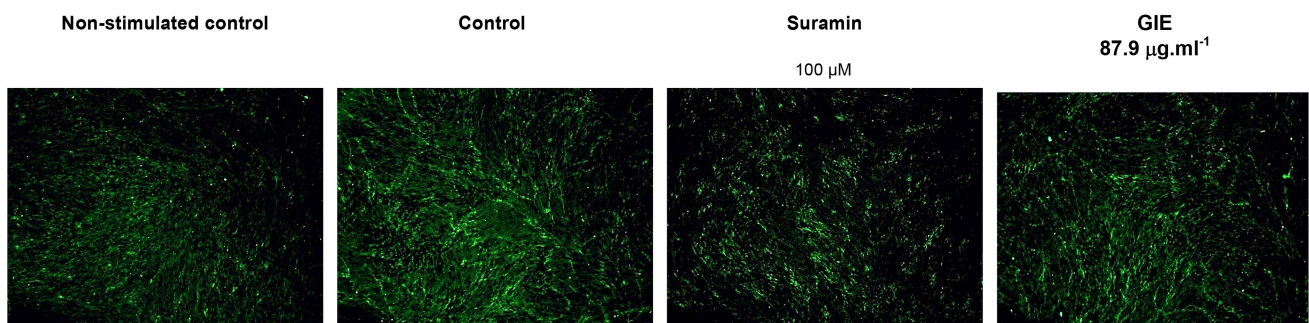
FIGURE 1 Percentage cell viability of NHDF or HMVEC-d cells after GIE treatment at different concentrations ( $\mu\text{g mL}^{-1}$ ). Values are presented as mean  $\pm$  standard error of mean (SEM) of triplicate experiments.

**FIGURE 2** VEGF-A release by fibroblast (NHDF). Values are presented as mean  $\pm$  standard error of mean (SEM).  $n = 3$ . NHDF were cultured for 24 h in culture medium. The medium was then replaced by culture medium containing or not (control),  $2.93 \mu\text{g mL}^{-1}$  GIE or PMA at  $5 \mu\text{g mL}^{-1}$  and the cells were incubated for 48 h, before VEGF-A quantification.  $**p < 0.01$ .

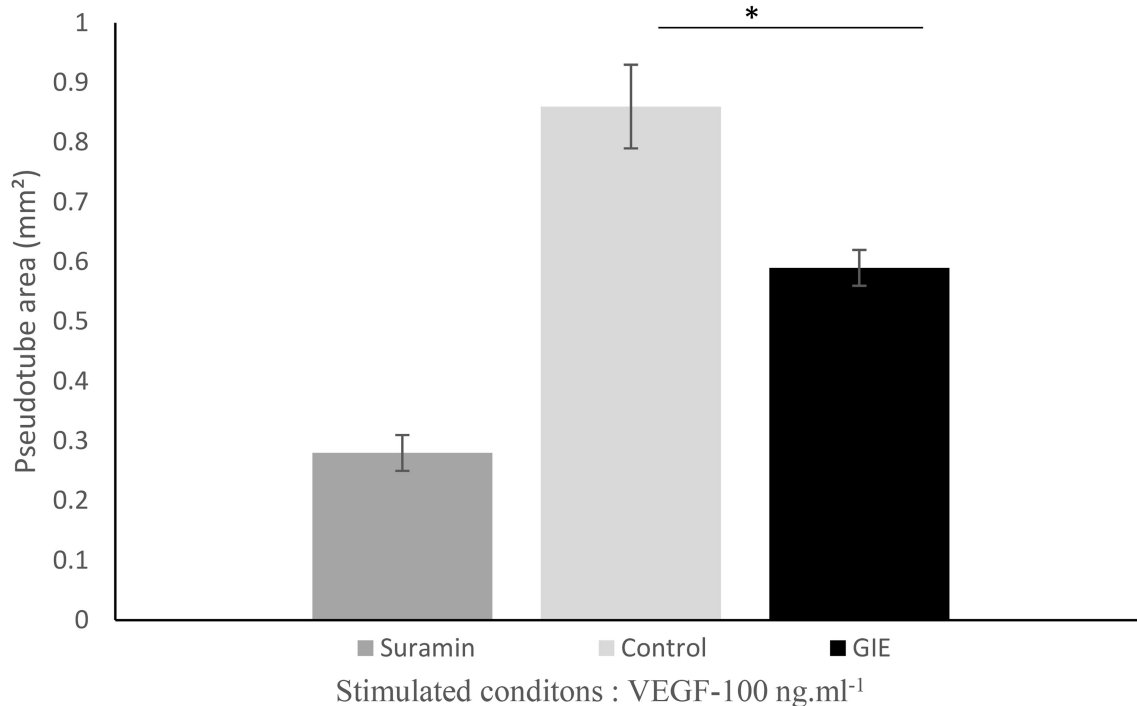


(a)

Stimulated conditions: VEGF – 100 ng/ml



(b)



**FIGURE 3** (a) Quantification of pseudotubes formation in co-culture of NHDF-HMVEC-d, after  $87.9 \mu\text{g mL}^{-1}$  GIE treatment in VEGF-stimulated conditions. Illustrative pictures of VWF immunostaining for each condition (C): VWF in green colour. (b) Effect of  $87.9 \mu\text{g mL}^{-1}$  GIE on the formation of pseudotubes in HMVEC-d-NHDF co-culture under VEGF-stimulated conditions. Values are presented as mean  $\pm$  standard error of mean (SEM). The HMVEC/NHDF co-culture was cultured for 24 h. The medium was then removed and replaced by co-culture medium containing or not (stimulated control),  $87.9 \mu\text{g mL}^{-1}$  GIE or the reference (suramin at  $100 \mu\text{M}$ ) and VEGF at  $100 \text{ ng mL}^{-1}$  was added. The cells were incubated for 7 days with treatment renewal after 72 h of incubation.  $n = 3$ .  $*p < 0.05$ .

## Ex vivo evaluation

### Effect of GIE on advanced glycation end product

In basal condition, exposition to  $147 \mu\text{g mL}^{-1}$  GIE significantly increased Glo-1 by 19% ( $p < 0.01$ ) when compared to control.

In UVA-stimulated conditions, Glo-1 was increased by 35% ( $p < 0.001$ ) as compared to UV-stimulated control. In situ visualization of Glyoxalase-1 is shown in Figure 4 as specific signal intensity range (green).

Concerning Carboxymethyl-lysine (CML), in UVA-irradiated condition, GIE treatment significantly reduced its expression by  $-31\%$  ( $p < 0.001$ ) as compared to UVA-stimulated control.

## Clinical data

### Dermatological and ophthalmological tolerance

In the dermatological evaluation, there were no reports of reactions to the gel cream. The ophthalmological evaluation indicated that none of the subjects presented a reaction related to the application of gel cream during this study. Thus, the global tolerance of the cream, assessed by the dermatologist, was concluded to be of 'excellent dermatological tolerance', whereby it was judged to be non-irritant, mild and suitable for sensitive skin.

### Efficacy end point

#### a. Measurement analysis related to upper eyelids

The acquisition software allowed to determine parameters of the cutaneous relief on 10 vertical profiles, corresponding to 10 lines across the upper eyelid within the zone of interest marked by a red square. The analysis was made on the average of the 10 profiles and allowed to calculate:

- Ra: Average roughness of the relief (in  $\mu\text{m}$ )
- Rz: Average relief (in  $\mu\text{m}$ )

$147 \mu\text{g mL}^{-1}$  GIE gel cream significantly reduced the average roughness (Ra) of the upper eyelid skin ( $-18\%$  vs. D0:  $p < 0.05$ ;  $-16\%$  vs. placebo:  $p < 0.05$ ) and the average relief (Rz) after 14 days ( $-22\%$  vs. D0:  $p < 0.01$ ;  $-17\%$  vs. placebo:  $p < 0.05$ ) (Figure 5a,b).

The average skin relief in the tear trough area was also significantly decreased ( $-6\%$  vs. D0;  $-17\%$  vs. placebo:  $p < 0.05$ ) after 14 days of GIE treatment.

#### b. Measurement analysis related to dark circles

Significant decrease in the redness of dark circles ( $-14\%$  vs. D0:  $p < 0.01$ ;  $-9\%$  vs. placebo:  $p < 0.05$ ) was reported after GIE treatment.

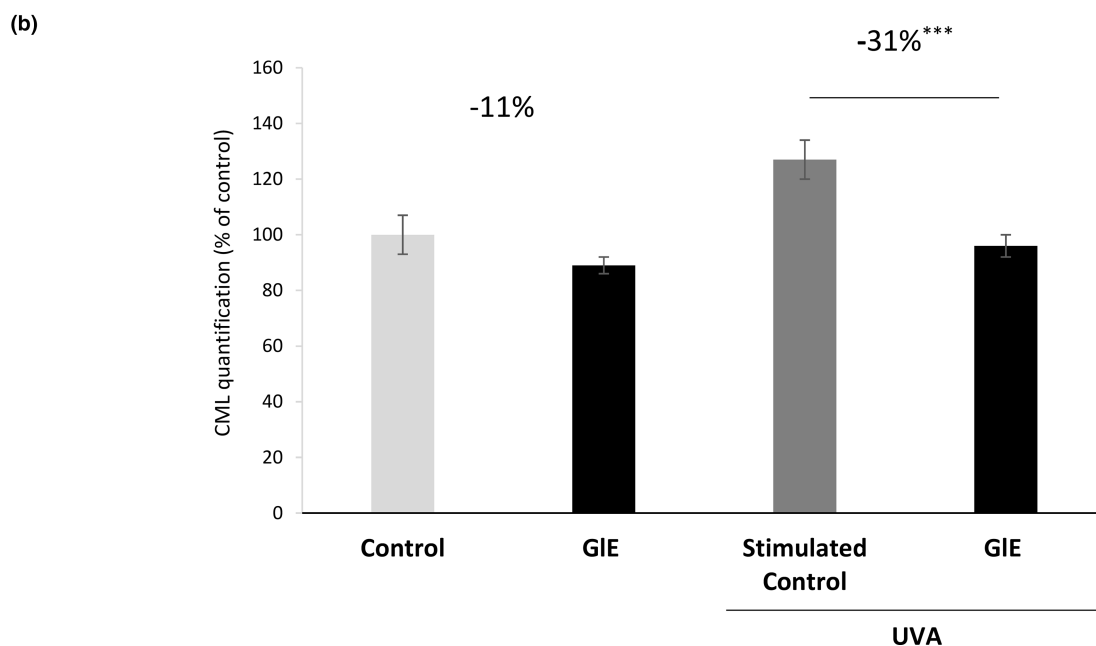
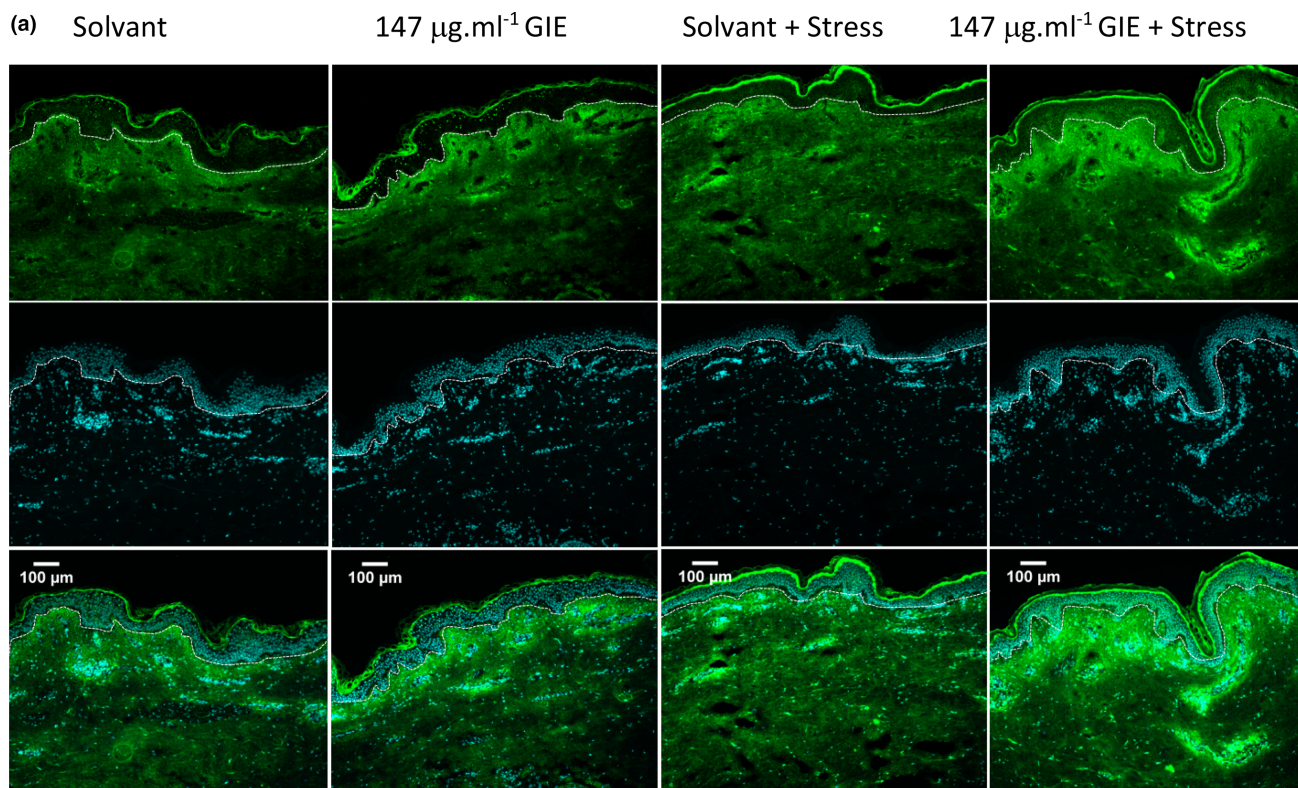
## DISCUSSION

Gentiopicroside (GP) has been investigated to exhibit pharmacological effect such as anti-inflammatory, antioxidant and smooth muscle relaxing effect [13]. GP has also been documented to reduce tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) levels. Liu et al. (2020) also showed GP was significant with the increase in antioxidant enzyme activity and antioxidative stress [9]. Using in vitro, ex vivo and clinical approaches, the aim of this investigation was to evaluate the potential of a Gentiopicroside-rich *Gentiana lutea* extract (GIE) to modulate skin around the eyes.

In vitro tests suggest that GIE has a significant positive effect on VEGF-C protein released by NHDF. VEGF-C is an important mediator of lymphangiogenesis. Lymphatic vessels are heavily involved in regulating the inflammatory response by draining extravasated fluid, antigens and it is well known that activation of lymphatic vessels induced by VEGF-C reduces oedema formation in acute skin inflammation [7].

It has been shown that ageing induces gradual atrophy and decrease in density and network complexity of the lymphatic system, promoting water accumulation in the tissues [14]. A total quantity of  $87.9 \mu\text{g mL}^{-1}$  GIE showed inhibitory properties on pseudotube formation induced by VEGF in the endothelial cell/fibroblast co-culture model (Figure 3a,b). One can put forward the hypothesis that GIE, through the activation of the VEGF-C/VEGFR-3 signalling pathway and the reduction release of chemotactic inflammatory mediators, may decrease eyelid oedema. These results also suggest that GIE would have a protective effect against excessive or dysregulated blood vessel formation and would ultimately protect against dark circles formation under the eyes. Overproduction of VEGF-A can be induced by AGEs, a group of modified proteins and/or lipids, which also up-regulate proinflammatory cytokines such as IL-6 via RAGE-NF-Kappa B pathway activation [15]. AGEs also increase reactive oxygen species formation and impair antioxidant systems. Among these AGEs is the glycoxidation product, CML, which is the only (with pentosidine)

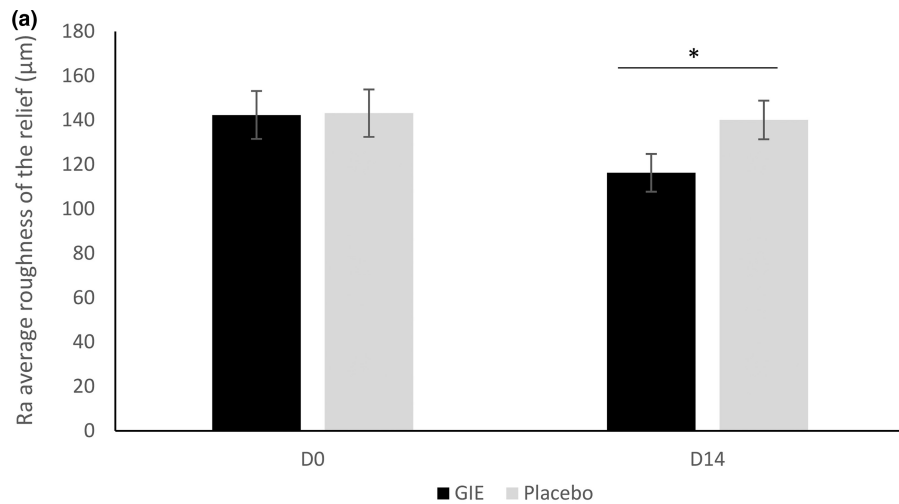




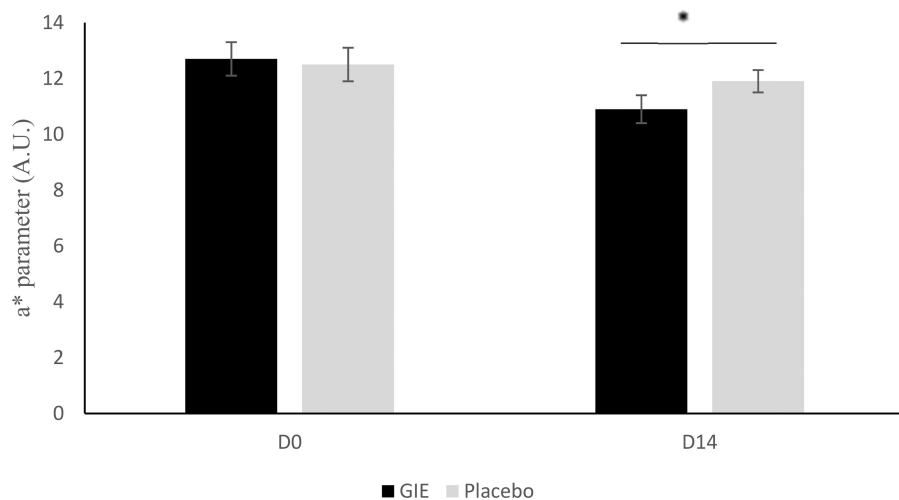
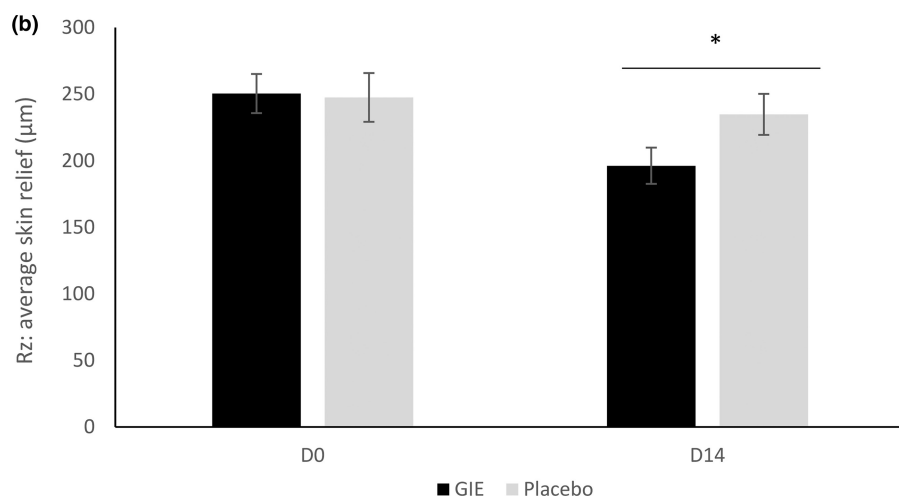
**FIGURE 4** (a) In situ visualization of Glyoxalase-I. The signal specific to Glyoxalase-1 labelling was obtained by epi-fluorescence microscopy (10× objective) and visualized (in green). Images of nuclei labelling (in cyan) and images of their overlay with Glyoxalase signals are also shown. White dotted lines separate the dermis, epidermis and stratum corneum skin compartments. (b) Signal of Carboxymethyl-lysine (CML) is presented in bar graphs by each experimental group (% of fluorescence intensity in relation to the explants treated with 147  $\mu\text{g}\cdot\text{mL}^{-1}$  GIE irradiated or not). Values are presented as mean  $\pm$  standard error of mean (SEM). \*\*\* $p < 0.001$ .

chemically characterized AGEs known to accumulate in protein with age [16]. It can be accumulated in tissues such as skin. In skin, glycation of *collagen I* induces the synthesis of CML in dermal compartments and epidermal compartments and, consequently, an ageing phenotype consisting

of poor stratification of epidermal layers and vacuolization of keratinocyte cytoplasm. To limit AGEs accumulation in tissues, detoxification mechanisms that eliminate AGE precursors involves the glyoxalase enzymatic system, such as Glo-1 [17]. Our results showed that topical 147  $\mu\text{g}\cdot\text{mL}^{-1}$



**FIGURE 5** (a) Ra parameter (acquisition using DermaTop®) reflecting the average roughness of the skin relief at D0 and after 14 days (D14) of treatment with placebo or  $147\mu\text{g mL}^{-1}$  GIE gel cream. Values are presented as mean  $\pm$  standard error of mean (SEM).  $n = 22$ . \* $p < 0.05$ . (b) Rz parameter (acquisition using DermaTop®) reflecting the average skin relief at D0 and after 14 days (D14) of treatment with placebo or  $147\mu\text{g mL}^{-1}$  GIE gel cream. Values are presented as mean  $\pm$  standard error of mean (SEM).  $n = 22$ . \* $p < 0.05$



**FIGURE 6** Skin colour characterization by  $a^*$  parameter at D0 and after 14 days (D14) of treatment with placebo or  $147\mu\text{g mL}^{-1}$  GIE gel cream.  $n = 22$ . Values are presented as mean  $\pm$  standard error of mean (SEM). \* $p < 0.05$ .

GIE increased Glo-1 by 19% ( $p < 0.01$ ) when compared to control. At the same time, the expression of CML was significantly reduced. Concerning the clinical evaluation,  $147\mu\text{g mL}^{-1}$  GIE extract compared to placebo showed systemic improvements in skin around eyes condition after 14 days (Figures 5 and 6).

## CONCLUSION

Perturbances in skin around eyes can lead to a prematurely aged appearance. By acting on the pathway of AGEs, VEGF-A and VEGF-C, GIE seems to allow a rejuvenation of the skin resulting, among other things, in a

decrease in redness. It now would be interesting to evaluate the efficacy of GIE on skin around eyes being established [18].

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## CONFLICT OF INTEREST STATEMENT

None.

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