A multiparametric, stepwise *in vitro* approach to identify anti-dark circle and anti-puffiness ingredient

Lorion Chloé, Lavastre Cindy, Rascalou Adeline, Lopez-Gaydon Amandine, Bonnet Sébastien, Rinaldi Thomas, Charton Virginie, **Vogelgesang Boris***, Bechetoille Nicolas,

Gattefossé SAS, Saint-Priest, France

* Corresponding author : Vogelgesang Boris, Gattefossé SAS 36 chemin de Genas 69804 Saint-Priest Cedex, +33 4 26 04 16 19, bvogelgesang@gattefosse.com

Abstract

Dark circles are a cosmetic concern worldwide, often associated with tiredness or aging. Owing to its thinness, the highly vascularized eye contour area easily shows blood and lymphatic circulation disorders. Environmental stress alters skin microcirculation and increases oxidation in the subocular area. The multifactorial nature of dark circles and puffiness represents a real challenge for *in vitro* efficacy testing of active ingredients.

Therefore, to select an active ingredient with both anti-puffiness and anti-dark circle potentials, we have implemented a screening strategy that combined different biological models addressing relevant targets of skin microcirculation and endothelial barrier function. Using this screening approach on 22 plant extracts, we identified a particularly interesting plant extract, which significantly reduced adhesion protein VCAM1 synthesis in human dermal microvascular endothelial cell (HDMEC) cultures, significantly decreased leukocyte adhesion to HDMEC membranes, while significantly increasing trans-endothelial electrical resistance (TEER). This ability to improve skin microcirculation was illustrated in a vascularized 3D dermis model stimulated with TNF-α, in which the plant extract was shown to restore the basement membrane of capillary-like tubular structures, as shown by laminin expression. Additionally, the extract also favored hemoglobin degradation by stimulating Heme Oxygenase-1 (HMOX-1) mRNA expression in dermal fibroblast cultures.

The stepwise selection model we used allowed us to identify a unique plant extract with promising anti-puffiness and anti-dark circle potential, based on combined proteomic, genomic and biochemical methods in acellular assays, 2D and 3D cell models. Dedicated clinical study will be used in the near future to demonstrate the *in vivo* benefits of the ingredient.

Keywords: dark circle; puffiness; microcirculation; endothelial barrier function

Introduction.

Dark circles under or around the eyes are a cosmetic concern worldwide, often associated with tiredness or aging. The pathophysiology of dark circles and puffiness is multifactorial, and nowadays, no detailed description is available in the literature. Skin under the eyes is very thin, about 0.5 mm thick (3 times thinner than on the rest of the face), and so extremely sensitive to environmental stress: UV, pollution, sleep deprivation. Owing to its thinness, and subsequent translucency, the highly vascularized and innervated eye contour area easily shows blood and lymphatic circulation disorders [1]. Dark circles and puffiness often co-exist and are aggravated with aging when skin slackens [2, 3].

Environmental stresses trigger a signaling cascade with the stimulation of cutaneous sensitive nerve fibers upstream, which release neuropeptides [4, 5] and induce skin inflammation [6]. Pro-inflammatory mediators secreted in the extracellular space of dermis stimulate dermal microvascular endothelial cells and alter skin microcirculation, and more specifically the endothelial barrier function [7]. Skin microcirculation plays an important role in the regulation of skin homeostasis, blood pressure, and inflammatory response, due to its proximity to the external environment (located less than 1 mm from skin surface) [8–11]. Microvascular endothelial cells are the major components of blood vessels. They are interconnected by cell-cell junctions and form a barrier between blood and the surrounding dermal tissue. The endothelial barrier is a dynamic structure that controls exchange of fluids and solutes, including plasma proteins as well as cells, in particular leukocytes. Leukocytes play a central role in innate immunity and inflammatory processes [12, 13]. Indeed, under inflammatory conditions, various pro-inflammatory mediators including TNF-α act on endothelial cells to increase vascular permeability. The endothelial barrier function is weakened, and the opening of intercellular junctions leads to extravasation of leukocytes followed by their accumulation in skin tissue, responsible for the appearance of puffiness under the eyes. At the same time, leakage of red blood cells is the primary cause of hyperpigmentation, characteristic of dark circles [14]. Leukocyte extravasation is a highly regulated process that involves the engagement of complex interactions between leukocytes and vessel endothelium, including selectins (P-selectin, E-selectin), integrins, and intercellular adhesion molecule (ICAM1), vascular adhesion molecule (VCAM1), junctional adhesion molecules (JAM-1/A/C) and platelet endothelial cell adhesion molecule (PECAM-1) [15–17]. These interactions are well-coordinated and are known to occur sequentially [18].

An increase in oxidation is also observed in the subocular area, due to the breakdown of hemoglobin, the main component of red blood cells, which releases toxic pigmented by-products (free heme, a red pigment, and ferrous ions). Therefore, stimulation of heme oxygenase-1 (HMOX-1) to eliminate toxic free heme [19, 20], and chelation of ferrous ions which oxidize and produce ROS (Reactive Oxygen Species) [21], constitute a complementary strategy to fight against dark circles formation.

The multifactorial nature of dark circles and puffiness represents a real challenge for *in vitro* efficacy testing of active ingredients because it is necessary to act at several levels of the signaling cascade to improve the clinical appearance of dark circles and puffiness.

Therefore, to select an active ingredient with anti-puffiness and anti-dark circle potential, we have implemented a screening strategy that combined different biological models addressing targets relevant to skin microcirculation and endothelial barrier function. Using this screening model, 22 plant extracts were compared, and results obtained allowed us to identify a particularly interesting plant extract. This ability to improve skin microcirculation was then confirmed on an 3D innovative dermis model including a microcapillary network, while expression of hemoglobin oxygenase 1 (HMOX-1) was measured in cultured fibroblasts obtained from eyelid surgery leftovers.

Materials and Methods.

Synthesis of VCAM1 in inflammatory conditions, in 2D cultures of HDMEC.

HDMECs (Lonza, Basel, Switzerland) were pre-treated for 18 h with the ingredients to test, then stimulated with TNF-α (R&D Systems, Minneapolis, USA) at 0.2 ng/ml and treated concurrently for another 6 h in the presence of the ingredients to test. Untreated cells were used as control. We used *in situ* immunostaining coupled with image analysis to measure VCAM1 synthesis. Immunolabeling was performed using monoclonal mouse anti-VCAM1 antibody, clone 1.4C3 (Fisher scientific, Illkirch, France), followed by IgG goat anti-mouse Alexa Fluor 594 antibody (Invitrogen/Fisher Scientific). The fluorescence was read using *Cytation 5* Cell *Imaging* Multi-Mode Reader (Biotek/Agilent, Les-Ulis, France) with appropriate filters. Fluorescence measurements by image analysis were performed on common acquisition parameters. Results were expressed as % relative to untreated cells. Values were expressed as mean +/- standard deviation over 3 experiments (n=3). The statistical test was the non-parametric Mann-Whitney test.

Adhesion of peripheral blood mononuclear cells (PBMC) to HDMEC 2D monolayer cultures in inflammatory conditions

HDMECs (Lonza) were pre-treated for 18h with the ingredients to test, then stimulated with TNF-α (R&D Systems) at 0.2 ng/ml and treated concurrently for another 4h in the presence of the ingredients to test. Untreated cells were used as a control. Besides, PBMCs (Etablissement Français du Sang) were labeled with calcein (Sigma-Aldrich, Saint-Quentin-Fallavier, France) for 1h before being put in contact with HDMECs, previously stimulated and treated. After 1h of PBMC adhesion, HMDEC monolayers were washed with saline phosphate buffer and adherent PBMCs were counted by measuring fluorescence using *Cytation 5* Cell *Imaging* Multi-Mode Reader (Biotek) with appropriate filters. Fluorescence measurements by image analysis were performed on common acquisition parameters. Results were expressed in % relative to untreated cells. Values were expressed as mean +/- standard deviation over 3 experiments (n=3). The statistical test was the non-parametric Mann-Whitney test.

TEER in inflammatory condition in 2D cultures of HDMECs

HDMECs (purchased from Lonza) were pre-treated for 24h with the ingredients to test, then stimulated with TNF-α (R&D Systems) at 0,2 ng/ml and treated concurrently for 24h in the presence of the ingredients to test. Untreated cells were used as a control. Integrity of the endothelial barrier was evaluated by measuring the TEER of the HDMEC layer (in Ohms) in real time using a calibrated system composed of 2 electrodes (Millicell ERS-, Millipore/Merck, Molsheim, France). Results were expressed in % relative to untreated cells. Values were expressed as mean +/- standard deviation over 3 experiments (n=3). The statistical test was the non-parametric Mann-Whitney test.

Laminin expression in a vascularized 3D dermis model in inflammatory conditions

Fibroblasts from juvenile foreskin (<2-year-old) and Human Umbilical Vein Endothelial Cells (HUVEC) were seeded ($0.75x10^6$ cells) onto Alvetex scaffold inserts and cultured for 28 days in a mixture of 2 media (50/50): Endothelial cell growth medium-2 (EGM2) (Promocell/Merck, Saint-Quentin-Fallavier, France) and Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone). This mixture was supplemented with 50 µg.ml-1 ascorbic acid (Sigma-Aldrich) and 10 ng.ml-1 EGF (AMS Bio) at 37°C in 5% CO_2.

The reconstructed dermis samples were treated with the ingredient to test and with TNF-α (R&D Systems), that were added in the culture medium respectively at 0,2% and 1 ng/ml, during the maturation of the dermal equivalent (from day 14 until day 28). Fresh active extract was added every two days. Untreated samples were used as control.

Samples of dermal equivalent were embedded in Tissue Tek OCT compound (Microm Microtech, Brignais, France), frozen in liquid nitrogen and cut to 5 µm thick sections (HM 560 Cryostat, Microm Microtech). Immunolabeling were performed with monoclonal mouse anti-CD31 (PECAM-1) antibody and polyclonal rabbit anti-laminin antibody (Sigma-Aldrich) followed by IgG goat anti-mouse Alexa 555 antibody and anti-rabbit Alexa 633 antibody. The mounting medium (Prolong Gold antifade reagent, Fisher scientific) contained DAPI for staining nuclei. Fluorescent immunostaining was observed using an Axio Imager M2 fluorescence microsocope (Zeiss, Rueil-Malmaison) and quantified (fluorescence area) using Zen image analysis software (Zeiss).

Transcriptomic expression of HMOX-1 in 2D cultures of human dermal fibroblasts

Adult normal human dermal fibroblasts (NHDF), obtained from eyelids of patients undergoing surgical procedure after inform consent in accordance with the Declaration of Helsinki, were treated for 6h with the ingredients to test. Untreated cells were used as a control. NHDF were washed with a saline phosphate buffer then lysed for RNA extraction. After RNA quantification and validation of quality control, HMOX-1 gene relative expression was evaluated using real-time PCR technique. Values were expressed as mean +/- standard deviation over 3 experiments (n=3). The statistical test was the non-parametric Mann-Whitney test.

***In tubo* chelation of ferrous ions**

The ingredients to test at increasing concentrations were put in contact with an iron chloride solution for 10 min. In order to evaluate the chelating power of the products, ferrozine (Sigma-Aldrich) was then added to the mixture for 10 min. In the presence of ferrous ions, ferrozine forms a complex of intense violet color. The absorbance of the solution can be measured at 562nm.The lower the absorbance of solution containing the extract is, the greater the chelating power of the product is.

Results.

At the beginning of our study, 22 different plant extracts were evaluated for their ability to reduce VCAM-1 expression, reduce PBMC adhesion, increase TEER, increase HMOX-1 and increase chelation of ferrous ions. For clarity, only results obtained with the most promising ingredient at the end of evaluation process are presented here. This ingredient was obtained from dried ground leaves using supercritical CO_2 added with ethanol as cosolvent for extraction. This ingredient is referred to as CO2/EtOH extract herein after.

Synthesis of VCAM1 in inflammatory conditions in 2D cultures of HDMEC.

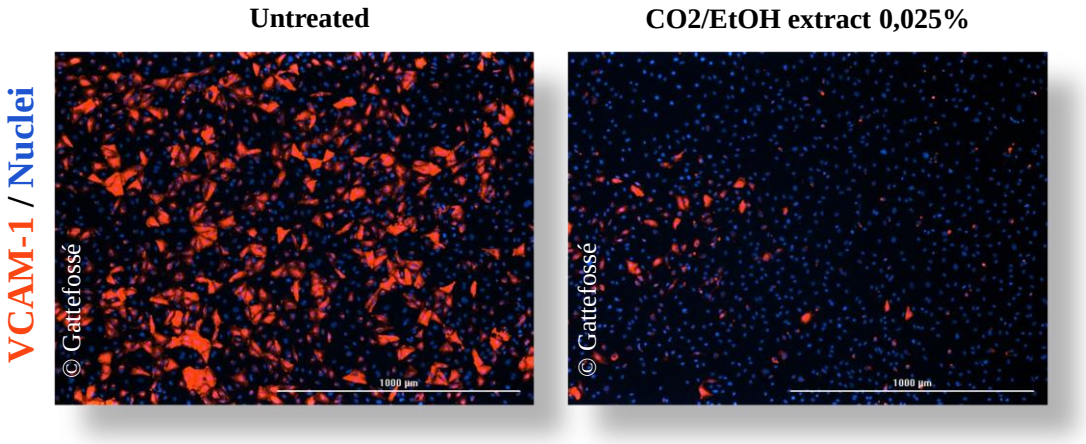

Figure1: Immunofluorescence imaging of VCAM1 synthesis in HDMEC cultures, in inflammatory conditions, treated or not with the CO2/EtOH extract at 0,025%. Scale bar = 1000µm.

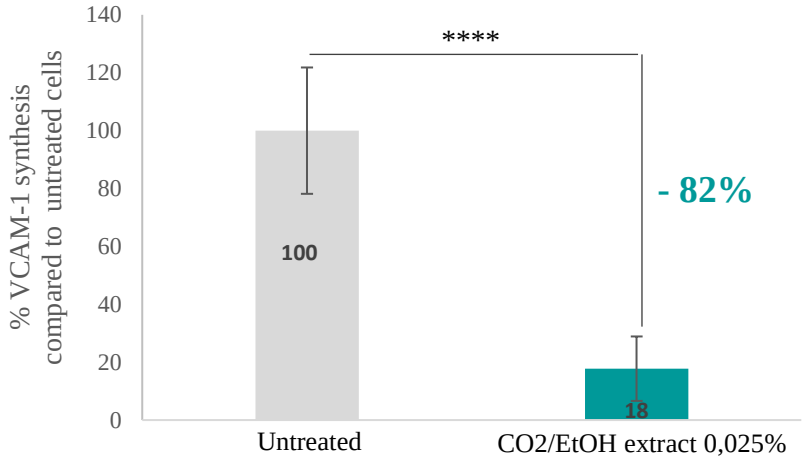

Figure 2: Immunofluorescence image analysis (analysis of 6 images per experiment, n=3 independent experiments). Mean VCAM1 fluorescence (in % compared to untreated cells) in HDMEC cultures, in inflammatory conditions, treated with CO2/EtOH extract at 0,025%. The statistical test was the non-parametric Mann-Whitney test, **** p<0,0001.

The CO2/EtOH extract at 0,025% induced a significant decrease in VCAM1 protein synthesis (-82% vs untreated) in HDMECs in inflammatory condition compared to untreated cells (Figures 1-2). The extraction solvent alone did not induce any reduction in VCAM1 synthesis (data not shown). Therefore, the extract extract decreased vascular permeability in inflammatory conditions, *i.e.* escape of white and red blood cells into the extracellular space and consequently the extract improved the endothelial barrier function.

Adhesion of peripheral blood mononuclear cells (PBMC) to 2D monolayer cultures of HDMEC in inflammatory condition

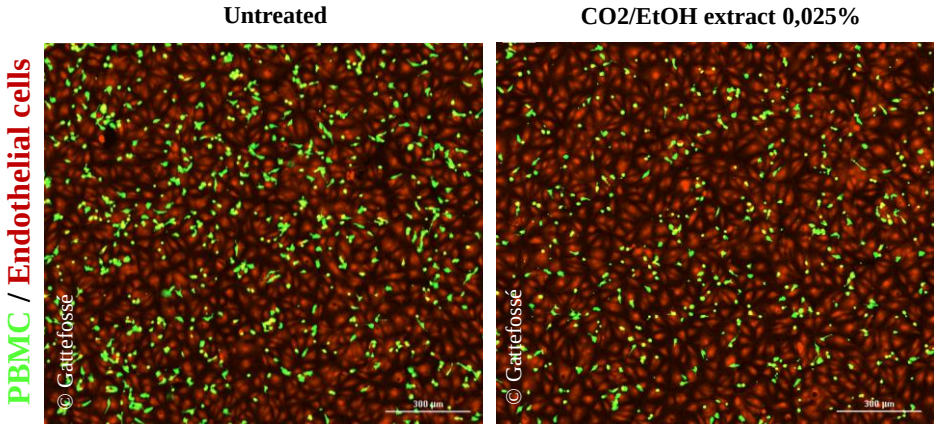

Figure3: Immunofluorescence imaging of PBMC adhesion to HDMEC membranes, in inflammatory conditions, treated or not treated with *CO2/EtOH extract* at 0,025%. Scale bar = 300µm.

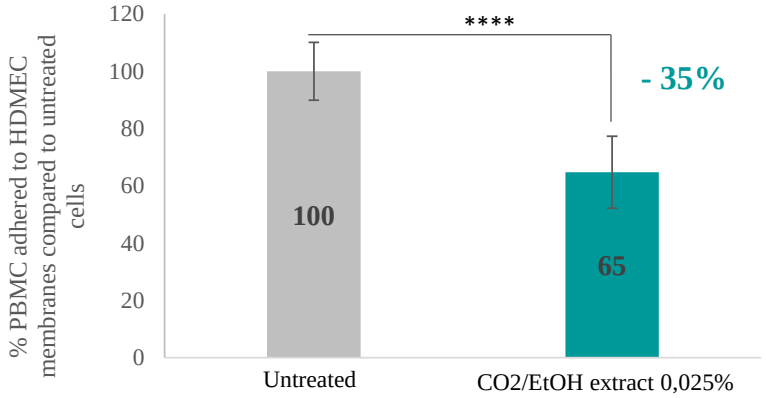

Figure 4: Immunofluorescence image analysis (analysis of 6 images per experiment, n=3 independent experiments). Mean PBMC fluorescence labeled with calcein which have adhered to HDMEC membranes, in inflammatory conditions, treated or not with CO2/EtOH extract at 0,025%. The statistical test was the non-parametric Mann-Whitney test, **** p<0,0001.

CO2/EtOH extract at 0,025% significantly decreased PBMCs adhesion (-35% vs untreated) to HDMEC monolayers under inflammatory conditions compared to untreated condition (Figures 3-4). The extraction solvent alone did not induce a decrease in adhered PBMCs number (data not shown). Therefore, CO2/EtOH extract decreased vascular permeability in inflammatory conditions, *i.e.* escape of white and red blood cells into the extracellular space. As a consequence, the extract was shown to improve the endothelial barrier function.

TEER in inflammatory conditions in 2D cultures of HDMECs

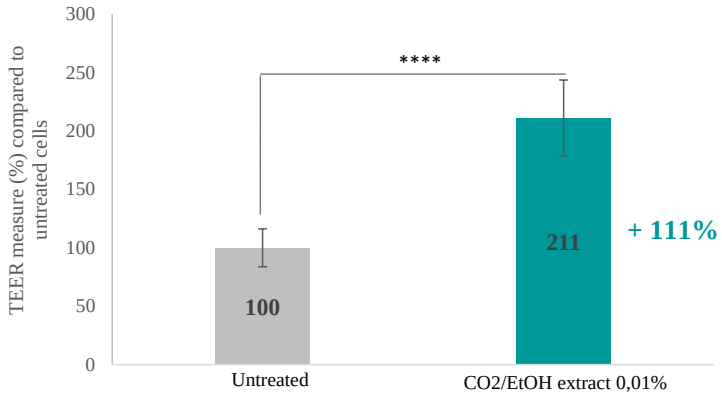

Figure 5: Measurement of TEER (mean of 3 independent experiments in % relative to untreated cells). TEER measure in HDMEC cultures, in inflammatory conditions, treated or not with CO2/EtOH extract at 0,01%. The statistical test was the non-parametric Mann-Whitney test, **** p<0,0001.

CO2/EtOH extract at 0,01% induced a significant increase in TERR (+111% vs untreated) in monolayer cultures in inflammatory conditions compared to untreated condition (Figure 5).

The extraction solvent alone did not induce an increase in TEER (data not shown). Therefore, CO2/EtOH extract increased the endothelial barrier function in inflammatory condition, and therefore it induced a decrease in vascular permeability.

CO_2/EtOH extract stimulated laminin expression in a vascularized 3D dermis model in inflammatory condition.

A vascularized 3D dermis model was treated with CO2/EtOH extract at 0.2% in inflammatory conditions during dermal maturation (from day 14 to day 28). Using immunofluorescence analysis, we showed that laminin expression was increased in dermal equivalents treated with CO2/EtOH extract compared to untreated samples.

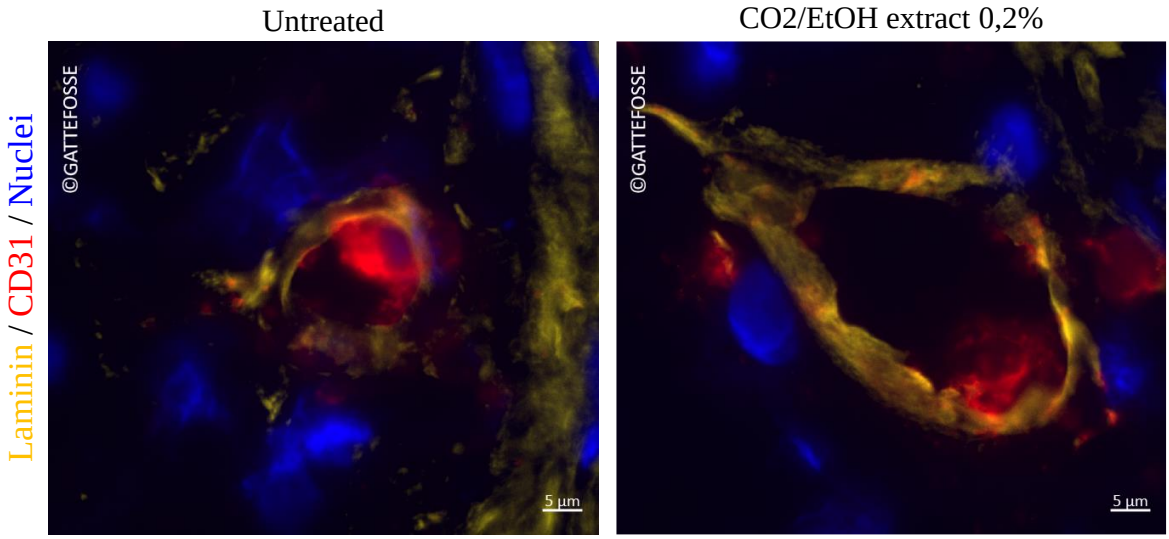

Figure 6: Vascularized 3D dermis model stimulated with pro-inflammatory cytokine TNF-α at 1 ng/ml, in which CO2/EtOH extract at 0,2% restored the basement membrane of capillary-like tubular structures, as shown by laminin expression (in yellow). Scale bar=5µm.

Transcriptomic expression of HMOX-1 in 2D cultures of human dermal fibroblasts

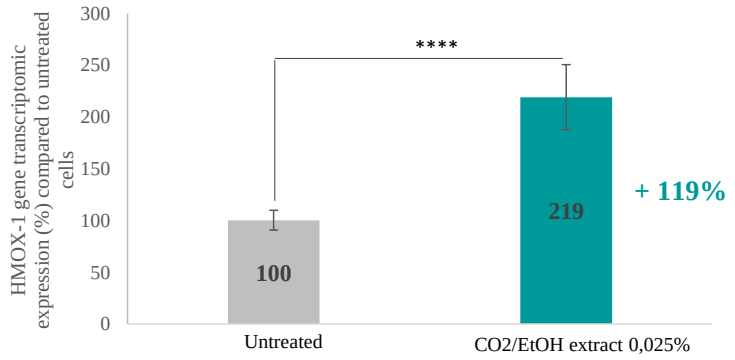

Figure 7: HMOX-1 gene transcriptomic expression (mean of 3 independent experiments in % relative to untreated cells). HMOX-1 gene expression in NHDF cultures treated or not with CO2/EtOH extract. The statistical test was the non-parametric Mann-Whitney test, **** $p<0,0001$.

CO2/EtOH extract at 0,025% induced a significant increase in HMOX-1 gene transcriptomic expression (+119% vs untreated) in NHDF cultures compared to untreated condition. The extraction solvent alone did not induce an increase in HMOX-1 gene expression (data not shown). Therefore, CO2/EtOH extract at 0,025% could be able to induce hemoglobin degradation, present in red blood cells, the primary cause of hyperpigmentation, characteristic of dark circles.

***In tubo* chelation assay**

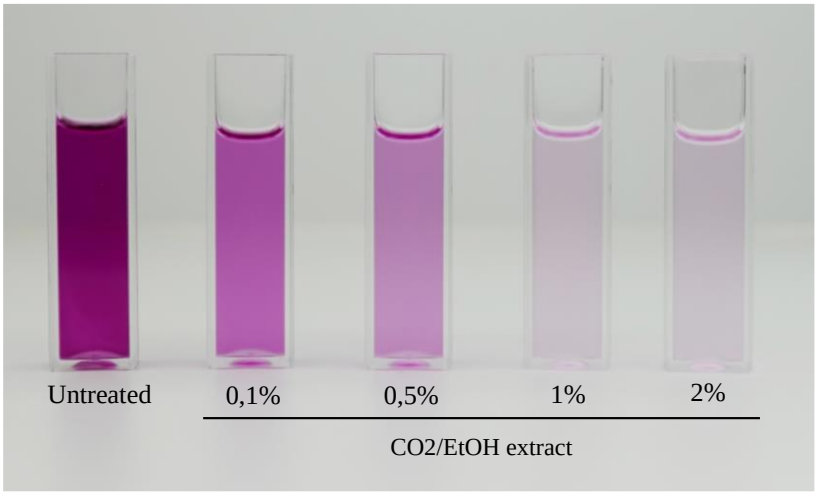

Figure 8: Chelation of ferrous ions (Fe^{2+}) evaluation, using an *in tubo* colorimetric assay, in a solution containing CO2/EtOH extract from 0.1% to 2% compared to untreated solution.

CO2/EtOH extract dose-dependently chelated ferrous ions whereas the extraction solvent alone did (data not shown). Therefore, CO2/EtOH extract could contribute to reduce

hyperpigmentation, characteristic of dark circles by chelating ferrous ions, which accumulate in the extracellular space due to hemoglobin degradation, the main component of red blood cells.

Discussion.

Dark circles and puffiness are the two most common aesthetic concerns of men and women around the world. Across cultures, societal perceptions are similar, in that level of fatigue and a person's age are preferentially based on periorbital aesthetics [22]. Therefore, it is necessary to reflect on a strategy to eliminate them. Nowadays, no detailed description of pathophysiology of dark circles and puffiness is available in the literature.

The appearance of dark circles is described as a physical or genetic disorder. While vascular dark circles are recognizable by their purple-blue color and are caused by a blood skin microcirculation disorder, pigmentary dark circles, yellow or brown in color, are usually of genetic origin linked to melanin overproduction (phototype IV) or generated by an overexposure to UV [23]. As for puffiness, it primarily results from a skin lymphatic microcirculation disorder, part of which is excess leukocyte extravasation, a process by which leukocytes can migrate between two endothelial cells. Leukocyte extravasation is a highly regulated process which involves complex interactions between leukocytes and endothelial cells through many adhesion molecules including VCAM1, selectins, integrins, the intercellular adhesion molecule (ICAM1), the junctional adhesion molecules (JAM-1/A/C) and the platelet endothelial cell adhesion molecule (PECAM1). Extravasation is facilitated by the sequential disruption of endothelial interactions, which form a paracellular space through which cells pass [16, 24]. Vascular endothelial (VE)-cadherin, a strictly endothelium-specific adhesion molecule located at endothelial cell junctions is of vital importance for the maintenance and control of endothelial cell contacts [25]. Mechanisms that regulate VE-cadherin-mediated adhesion are thus important for the control of vascular permeability and leukocyte extravasation [26] Measuring Tran-Epithelial Electrical Resistance is a simple way to measure cohesion of endothelial cells *in vitro*, and was thus integrated into our screening strategy.

Endothelial laminin isoforms can also affect leukocyte transmigration directly and indirectly. Laminin 511, for example, stabilizes junctional VE-cadherin and endothelial barrier function [27, 28]. We also observed laminin production a 3D model of dermis integrating endothelial cells.

As regards the pigmentary component of dark-circles, there is increasing evidence that HMOX-1 has a key role to play through its cytoprotective, antioxidant and anti-inflammatory abilities [29]. It catabolizes heme degradation into biliverdin (green pigment), then transformed into bilirubin (yellow pigment) by the action of biliverdin reductase A. These catabolites, known for their antioxidant roles, help to reduce dark circle appearance [30, 31]. However, free heme degradation also results in the release of iron molecules and their accumulation in skin tissue. Free ferrous ions oxidize and produce ROS, leading to increased skin oxidation and inflammation [32].

Ultimately, the multifactorial nature of dark circles and puffiness represents a real challenge for *in vitro* efficacy testing of active ingredients. Therefore, to select an active ingredient with both anti-puffiness and anti-dark circle potentials, we have implemented a screening strategy that combined different biological models addressing relevant targets of skin

microcirculation and endothelial barrier function. For this, we combined a semi-quantitative measurement of VCAM1 protein with more advanced functional assays, such as adhesion of PBMCs on endothelial cells, to evaluate the ability of ingredients to improve the endothelial barrier function.

Using this approach, we identified a unique CO2/EtOH plant extract that was able to significantly decrease VCAM1 protein synthesis (-82% vs untreated) in HDMEC cultures in inflammatory conditions. Moreover, the extract significantly decreased PBMCs adhesion to HDMEC under inflammatory conditions (-35% vs. untreated). This efficiency was reinforced by the ability of the extract to increase TEER (+111% vs untreated) in inflammatory conditions in 2D cultures of HDMECs.
Results previously obtained on skin microcirculation and endothelial barrier function were illustrated in a vascularized 3D dermis model challenged with TNF-α, in which the plant extract was shown to restore the basement membrane of capillary-like tubular structures, as observed by laminin expression.

To more specifically address dark circles, especially the degradation of free heme, we have shown that the CO2/EtOH extract was able to increase HMOX-1 gene expression (+119% vs. untreated) in fibroblast cultures obtained from eyelids surgery. Finally, we have also demonstrated that the extract was able to chelate ferrous ions in a dose-related manner.

Conclusion. The stepwise selection model we used allowed us to identify a unique plant extract with promising anti-puffiness and anti-dark circle potential, based on combined proteomic, genomic and biochemical methods using acellular assays as well as 2D and 3D cell models. Dedicated clinical study should be used in the near future to demonstrate the *in vivo* benefits of the ingredient.

Conflict of Interest Statement. NONE.

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