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"IN&OUT strategy for addressing psychological factors impacting skin physiology"

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1. Introduction

Stress is key to survival, finely regulated by the body and characterized by physiological reactions in response to environmental changes. Acute stress is a natural, essential, and beneficial reaction. When environmental challenges persist, or the individual can no longer cope, stress becomes chronic which can have a negative impact on the body. These may include sleep disturbance, cardiovascular disease, inflammatory diseases, and accelerated aging [1].

The complex interplay between the skin and the mind is well-documented, with psychological stress impairing skin homeostasis through mechanisms such as inflammation, barrier disruption, and altered cellular responses [2,3].

Stress is regulated through two primary physiological pathways: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS) [4]. Activation of the HPA axis by psychological stress initiates the release of corticotropin-releasing hormone (CRH) from the hypothalamus, which stimulates the secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland. ACTH subsequently promotes the synthesis and release of glucocorticoids, primarily cortisol, from the adrenal cortex [1]. While glucocorticoids exert potent anti-inflammatory effects during acute stress, their prolonged elevation under chronic stress conditions can suppress immune function and provoke inflammatory responses [5]. Simultaneously, the SNS is activated, leading to the release of catecholamines like adrenaline and noradrenaline.

The skin, serving as both a physical barrier and a stress-responsive organ, is directly influenced by these systemic stress pathways. Studies have demonstrated that the skin is particularly sensitive to stress, involving immune cells, hormones, and neurotransmitters [6-8]. Psychological stress is known to cause dermatoses associated with skin barrier defects, such as pruritus, psoriasis, or atopic dermatitis [2,9,10]. Choe et al. showed a higher level of cortisol in the *stratum corneum* of individuals under psychological stress (students in exam period) versus controls (students outside exam period). They also observed an increase in basal transepidermal water loss (TEWL) in stressed individuals, reflecting an alteration in the cutaneous barrier [11]. Stress has also been associated with oxidative stress, inflammation, immunity our wound healing disorders and diminished barrier function and dermal density [12]. Together, these adverse effects cause dry skin, associated with itching and loss of skin elasticity [13, 14].

The therapeutic use of plants for managing both skin and stress-related conditions is well established, suggesting their potential relevance in the treatment of stress-induced skin disorders [5]. Among those plants, Holy basil (tulsi, *Ocimum sanctum*) presents a unique combination of pharmacological actions that promote wellbeing. In the Ayurveda system, tulsi is often referred as an “Elixir of Life” for its healing powers and has been known to treat many different common health conditions [1]. This traditional medicine herbal plant demonstrates anti-oxidant, anti-microbial, and anti-tumor effects (reviewed by Kumar et al. [5]). It is commonly used in products, used as nutraceutical, aimed at stress relief and sleep improvement. A study showed that holy basil extract improved symptoms of general anxiety disorder, including anxiety, stress, depression and attention span after 60 days of treatment [15]. Holy basil has been found to lower elevated cortisol levels and regulate blood sugar, which can help normalize sleep cycles disrupted by stress (reviewed by Jamshidi et al. [16]). Besides its well-described action on stress, holy basil extracts has shown in an *in vitro* study inhibitory activities against oxidation, inflammation, collagenase, elastase, and hyaluronidase which may be of interest in addressing skin-aging issues [17].

Considering the previously published data on the beneficial effects of Tulsi on stress and skin, we have set-up a combination of two extracts to address stress related skin disorders by a topical application and a dietary supplement. To study their ability to improve skin conditions under stress, we evaluate its potential effects on 2D and 3D models exposed to various stress-mimicking factors which can be led to decipher the protective properties of the proprietaries ethanolic extracts of O. sanctum (EEOS). A clinical study has been conducted for the topical application of O. sanctum extract and the study on dietary supplement is currently made.

2. Materials and Methods

Cell culture and treatments

NO synthesis - Primary NHEK were seeded in a 96-well plate. Two days after, the spontaneous NO release was measured. Vehicle, EEOS (0.001 or 0.005%) or ascorbic acid (1mM) is then added with 1µM of cortisol and NO production was monitored for 10 minutes by using a specific amperometric probe. Data are expressed as delta amplitude of NO release. Statistical analysis : two-way ANOVA followed by Bonferroni’s post-hoc test.

Protein carbonylation, inflammation and cortisol synthesis - Primary NHEK were seeded in a 96 or 6-well plate and were cultured in appropriated medium at 37 °C in 5% CO_2 humidified air. NHEK were cultured for 4 days in the presence of stress factors: epinephrine (1 µM), neuropeptides αCGRP (1 µM), and substance P (10 µM). Cortisone (10µM) was added to stress condition as a substrate for cortisol formation. In parallel, EEOS was applied at 100 µg/mL and 300 µg/mL (vehicle ethanol at 70%). The timolol beta-blocker was used as a positive control reference to block epinephrine action. Oxidatively damaged (carbonylated) proteins were labeled using a fluorescent probe functionalized to specifically bind to carbonyl moieties and DAPI for nuclear labeling in PBS. Protein carbonylation and anti-inflammatory activity were assessed by measuring the fluorescence emitted by proteins specifically labelled on the cell layer. Keratinocytes were incubated with diluted anti-TSLP antibody (Abcam, ab47943) with the secondary antibody coupled to a fluorophore (Invitrogen, A21244). Cellular nuclei were labelled with DAPI. Fluorescent images were collected with an epifluorescent microscope and analyzed with ImageJ software. Inflammatory cytokines (Ella, Bio-Techne, San José, USA) and cortisol (ThermoF, EIAHCOR) production were assessed in collected supernatant and evaluated by ELISA according to the manufacturer’s guidelines.

Reconstructed full thickness human skin model

A reconstructed full thickness skin model was obtained by culturing NHDF in a scaffold matrix made of chitosan, cross linked collagen, and glycosaminoglycans during 21 days under optimized cell culture conditions for ECM neo-synthesis. NHEK were then seeded on the dermal equivalent constructs and raised at the air/liquid interface on day 28 to allow the formation of the epidermal compartment. To mimic the natural circulation of glucocorticoids under stress, betamethasone (a cortisone derivative) was applied to the medium for 2 days between days 43 and 44 of culture. EEOS was applied to the culture medium according to 2 distinct application protocol. A “preventive” protocol, during which 300µg/mL of EEOS was applied for 10 days : 8 days before stress and during the stress. Ethanolic solvent, used for EEOS solution, was used as a control. Treatments were renewed three times a week. Reconstructed skin models were recovered at day 46 of total cell culture and immediately fixed in neutral buffered formalin 4% (Diapath, Martinengo, Italy) and embedded in paraffin or in OCT compound and frozen at -80°C, for histological and immunohistological analysis, respectively. For each cell culture condition and analysis, 3D skin equivalents were produced in triplicate.

Histology, fluorescence staining, and immunostaining

Paraffin-embedded formalin-fixed samples were cut into 5µm sections. After dewaxing and rehydration, sections were stained with Harris’ hematoxylin-phloxin-saffron (HPS) for routine histology and modified Masson’s Trichrome light green variation to visualize collagenous connective tissue fibers.

For immunofluorescence, after heat-mediated antigen retrieval treatment, non-specific binding was blocked in PBS/BSA. Sections were then incubated with the following primary antibodies: claudin-1 and elastin diluted in PBS/BSA overnight at room temperature. After incubation for 1h with an AlexaFluor-488 or 568-conjugated anti mouse/rabbit secondary antibody (Molecular Probes, Invitrogen, France), nuclear counterstaining using 4’,6-diamidino-2-phenylindole (DAPI) was carried out routinely.

Clinical study

A double-blind placebo-controlled linical study has been performed on 40 to 60 years old Caucasian women (23 subjects in both O. sanctum leaf extract and placebo groups). O. sanctum leaf extract and placebo have been applied twice a day during 56 days on the face.Anti-ageing effects on the face were evaluated on wrinkles using analysis of topography ac-quired with Dermatop® (Eotech), on skin biome-chanical properties using Cutometer (Courage & Khazaka) and on skin complexion homoge-neity by image analysis acquired with Colorface® (Newtone Technologies).

Difference between O. sanctum leaf propanediol extract and placebo has been assessed by statistical analysis. Normality of the data was tested using a Shapiro-Wilk test, and a Mann-Whitney U or a non-paired Student’s t-test was used to assess the statistical significance of differences between O. sanctum leaf extract and placebo groups. p-value <0.05 was considered to indicate a statistically significant difference (noted ** on graphs) and p-value <0.1 was considered to indicate a statistically limit significant difference (noted * on graphs).

3. Results

a) *In vitro* evaluation

Cortisol induced NO release by NHEK and epinephrine, neuropeptides and cortisone stress induced cortisol synthesis by NHEK.

We have shown *in vitro* that EEOS (ethanolic extract of *Ocimum sanctum*) extract protects skin cells from stress related disorders.

EEOS significantly inhibited the production of nitric oxide by NHEK stimulated by cortisol (Fig.1a). On NHEK stimulated by epinephrine, neuropeptides and cortisone, EEOS tested at 300µg/mL significantly inhibited the production of cortisol by -11% (*p<0.05*)(Fig.1b).

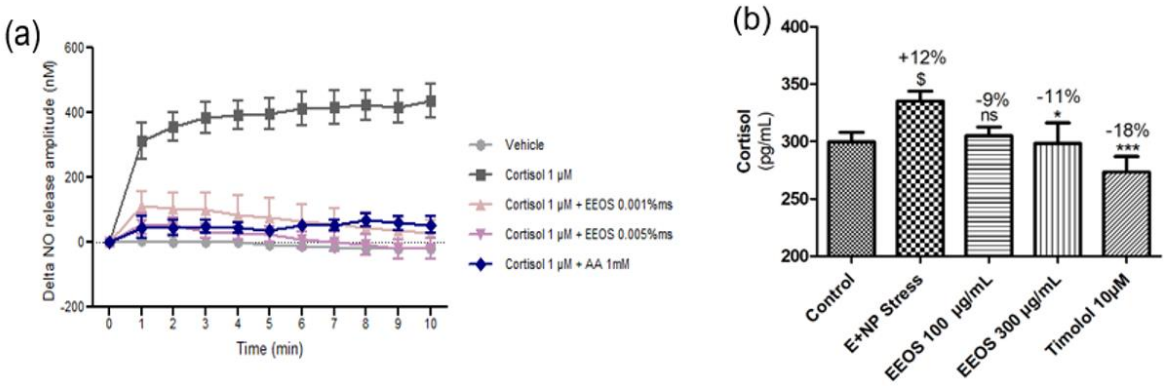


Figure 1 : (a) Quantification of NO release by NHEK stimulated by cortisol in presence or absence of EEOS or AA (ascorbic acid). (b) Quantification of cortisol synthesis by NHEK stimulated by epinephrine and neuropeptides (E+NP) and cortisone. $ p<0.05 vs control, ***p<0.001, *p<0.05 vs E+NP stress, one way ANOVA followed by post-hoc Tukey's test.

Epinephrine and neuropeptides stress induced an inflammatory state on NHEK with a significant increase of IL-1β (Fig.2a) and IL-8 (Fig.2b).

EEOS significantly inhibited the synthesis of the pro-inflammatory cytokines IL-1β and IL-8 on NHEK stimulated by epinephrine and neuropeptides stress (Fig.2a and Fig.2b).

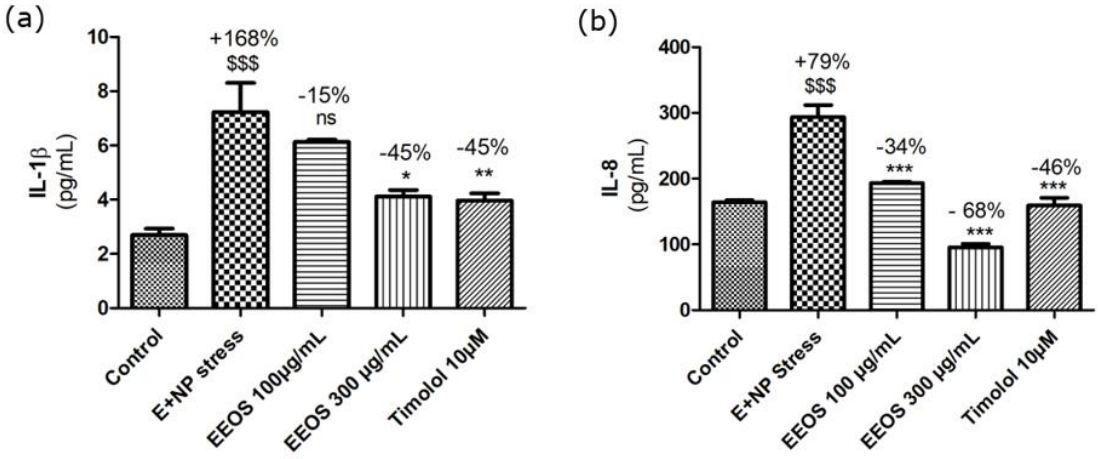


Figure 2 : Quantification of cytokines IL-1 β (a) and IL-8 (b) synthesis by NHEK stressed by an epinephrine and neuropeptides mix (E+NP). $$$ p<0.001 vs control, *p<0.05, ** p<0.01 and *** p<0.001 vs E+NP stress, one way ANOVA followed by post-hoc Tukey's test.

On reconstructed skin model, betamethasone stress led to a significant decrease in epidermal thickness and loss of claudin-1 compared to control. EEOS applied at 300µg/mL according to preventive and curative protocol significantly reversed the stress-induced reductions (Fig.3).

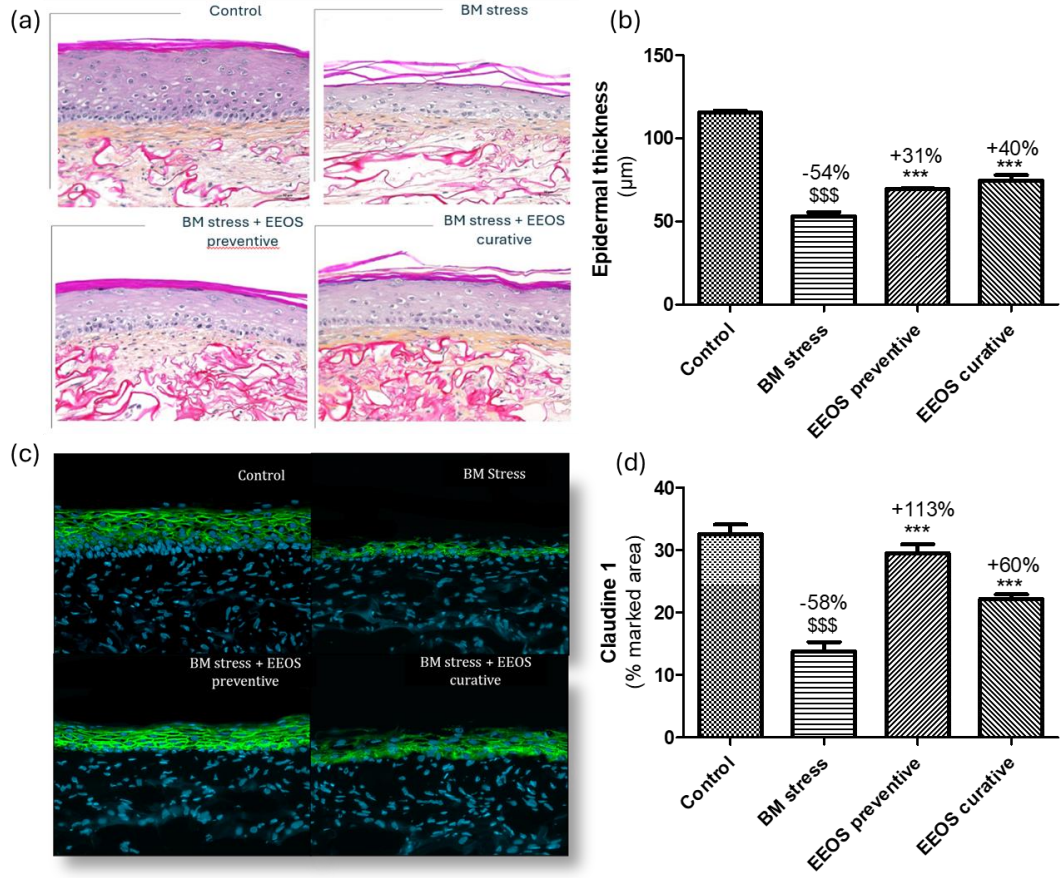
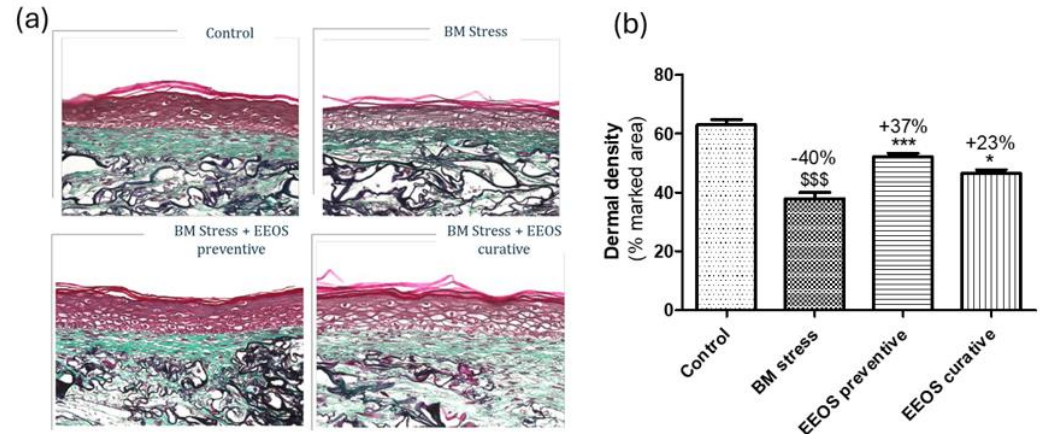


Figure 3 : (a) Illustration of hematoxyllin coloration on reconstructed skin and (b) quantification of epidermal thickness. Claudin-1 fluorescence staining on reconstructed skin (c) and its quantification (d). $$$ p<0.001 vs control, ***p<0.001 vs betamethasone (BM) stress, one way ANOVA followed by post-hoc Tukey's test.

Betamethasone stress significantly decreased the dermal density and elastin compared to control. EEOS applied systemically, according to a preventive or curative protocol significantly reversed the loss of dermal density and elastin density impacted by betamethasone stress (Figure 4).



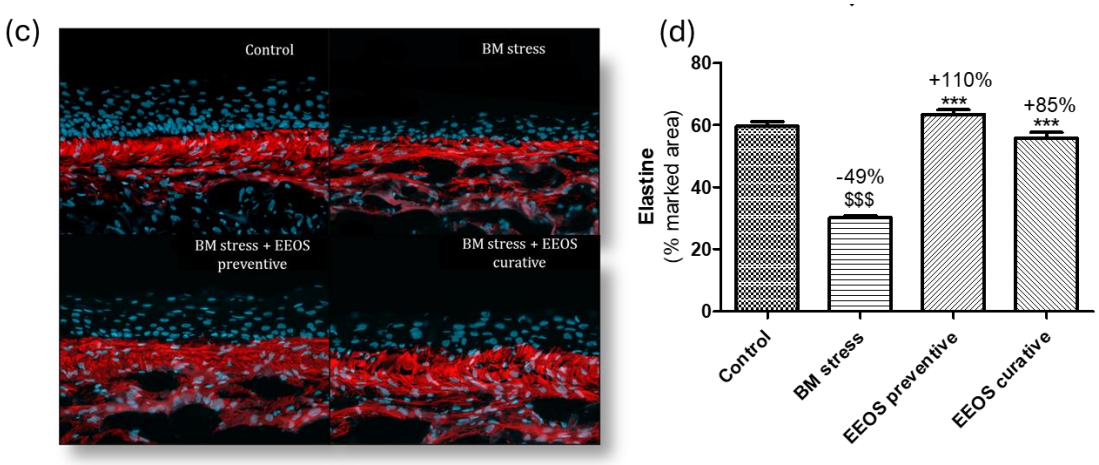


Figure 4 : (a) Illustration of masson's trichrome coloration on reconstructed skin and (b) quantification of dermal density. Elastin fluorescence staining on reconstructed skin (c) and its quantification (d). $$$ p<0.001 vs control, ***p<0.001, *p<0.05 vs betamethasone (BM) stress, one way ANOVA followed by post-hoc Tukey's test.

b) Clinical study

Propanediol extract of *Ocimum sanctum* showed significant anti-aging effects compared to placebo. It reduced significantly crow's feet wrinkles area and length (Figure 5). Skin elasticity and tonicity, key players in the biomechanical properties of the skin, were improved by *O. sanctum* application during 56 days (Figure 6). Finally, the extract improved the skin complexion homogeneity (limit significant) (Figure 7).

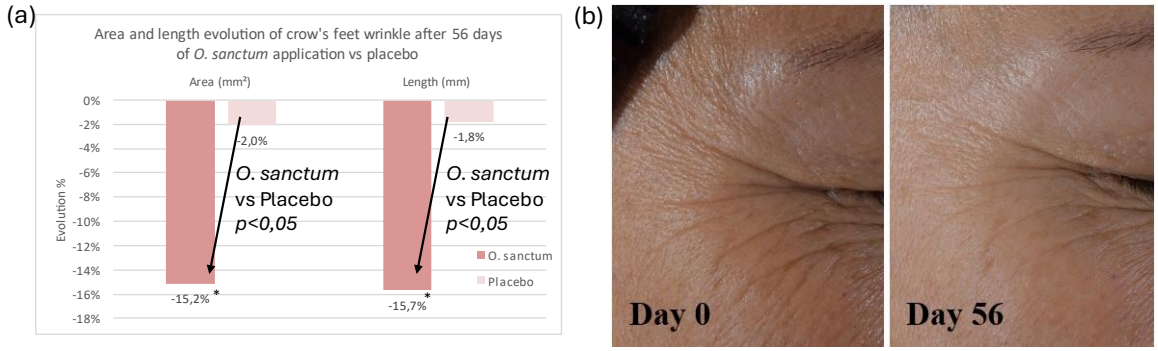


Figure 5 : Analysis of topography of crow's feet wrinkles (a) and illustration of crow's feet improvement between day 0 and day 56 (b). * p<0.01 vs day 0 (Student T Test) and p<0.05 vs placebo with Mann-Whitney U test.

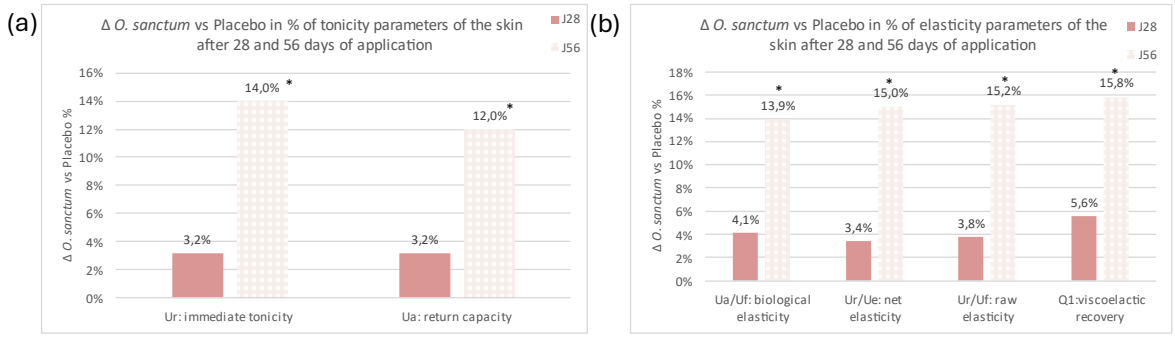


Figure 6 : Analysis of biomechanical properties by cutometry. *p<0.05 Student T test

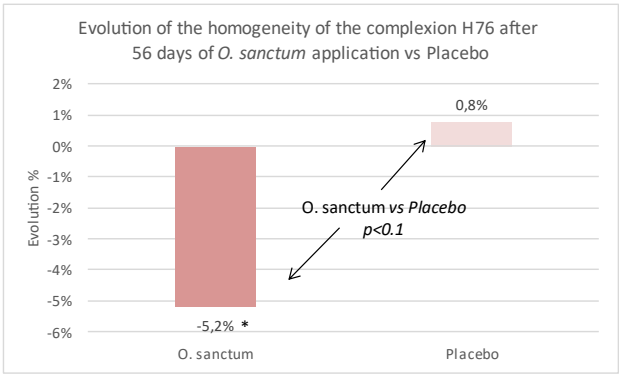


Figure 7 : Analysis of skin tone homogeneity at days 56. p<0.1 Student T test

4. Discussion

Holy basil has a long history of use in Ayurvedic traditional medicine and is gaining recognition in modern healthcare [5]. It his know to have antimicrobial, anti-inflammatory, antioxidant, and immunomodulatory effects. Holy basil has been described as having beneficial effects on stress and stress-related symptoms by helping the body respond to and manage stress more effectively [15,16].

Stress and skin conditions exacerbate each other in a vicious circle. Stress hormones, such as glucocorticoïds and catecholamines, can weaken the skin's immune defenses, trigger allergic responses, delay healing, and disrupt the skin's natural protective barrier [18]. Moreover, the link between psychological stress and aging is widely described with an acceleration of cellular senescence, a decrease of life expectancy and an increase of age-related pathology [19]. Chronic stress deregulates cortisol levels and their oscillation rhythm, resulting in non-physiological exposure [3,11]. Stress can increase overall inflammation in the body, which may have dermatological manifestations notably by pro-inflammatory cytokines like thymic stromal lymphopoetin (TSLP)[18,20]. Moreover, stress activates the nervous system, which can lead to increased sensitivity and reactivity in the skin [3]. These stress-induced changes can lead to various dermatological symptoms, such as eczema or psoriasis, that may increase psychological distress. These include visible symptoms causing embarrassment that can also have a social impact, physical discomfort [18]. A study on mouse model also described the link between chronic stress and skin aging with a contribution of glucocorticoids and catecholamines to the loss of collagen deposition and an increase in oxidation. [19]

During psychological stress, alteration of the epidermis and its barrier function are recognized targets [11]. By reducing epidermal thickness and organization, psychological stress leads to reduced hydration and impaired barrier homeostasy resulting in a loss of protection against

external agents [18,21-23]. Stress impacts the dermis with a decrease of collagen and elastin deposition and an increase of inflammation and oxidation [3,19].

The full thickness human skin model stressed by glucocorticoïds allows us to confirm the decrease of epidermis thickness, crucial markers of epidermal homeostasis and dermal extracellular matrix components. Among them, filaggrin and loricrin are essential proteins for stratum corneum integrity and claudin-1 is a marker of epidermal tight junctions, which ensure the cohesive structure of the epidermis, water permeability, and maintenance of hydration [24,25]. We report that EEOS successfully reversed the negative effects of betamethasone-induced stress, including epidermal thickness, epidermal barrier differentiation markers (loricrin (not shown) and claudin-1), and also observed a positive trend observed for filaggrin and epidermal hyaluronic acid (data not shown). In the same way, EEOS improved dermal density and elasticity (type I collagen, elastin and dermal hyaluronic acid-data not shown). These results confirm at the proteomic levels on 3D model some of the in tubo and 2D effects observed by Chaiyana et al (2019) regarding the inhibition of matrix proteases by Ocimum sanctum extract.

Our *in vitro* models on NHEK exposed to epinephrine and neuropeptides or cortisol allows us to study epidermal cells response. Quantification of cortisol production, as measured by ELISA in NHEK following exposure to epinephrine, neuropeptides and cortisone, showed that EEOS normalized keratinocyte cortisol synthesis to control levels. In the event of exogenous stress or psychological stress, an increased inflammatory state can be harmful to the skin's equilibrium [9, 18]. Our studies in NHEK further showed that EEOS reduced the increase in TSLP (data not shown), NO and inflammatory cytokines (IL-8 and IL-1β) caused by addition of epinephrine and neuropeptides or cortisol to the culture medium. The effect of EEOS was found to be dose-dependent on each target. Our model was inspired by the study of Dini et al. (2021) which presents the impact of epinephrin on oxidation and inflammation processes [26]. We refined the model by incorporating neuropeptides to better represent the complexity of the biological response to stress. Our data and data from Dini et al. (2021) revealed that this type of stress on keratinocytes could be a good way to evaluate the protective effects of plant extracts or other type of active ingredients.

In the clinical trial, Holy basil extract applied topically significantly improved skin appearance by decreasing crow's wrinkles and forehead wrinkles (data not shown) with a tendency to improve skin complexion homogeneity. Skin biomechanical properties like elasticity and tonicity has also been significantly improved. Skin appearance has a great impact on social perception and it is recognized that social perception is directly correlated with well-being [28]. Self-perception as well as social perception could impact the level of stress and as said before: stress and skin conditions exacerbate each other in a vicious circle.

Taken together, these information allow us to conclude that combining topical and oral approach is primordial to address stress related skin disorders. Enhancing skin appearance significantly influences perception, while mitigating the negative effects of stress on the skin can help delay aging and prevent skin disorders that may alter how one is perceived. A complementary clinical trial is currently done to study the efficiency of oral supplementation with our extract on stress regulation and stress related skin disorders.

5. Conclusion

To interrupt the vicious circle of stress and skin conditions, a comprehensive approach is necessary. Lifestyle modifications (e.g., improved diet, adequate sleep) can support both stress reduction and skin health. Our ethanolic extract of *Ocimum sanctum* greatly enhances skin appearance and key components of skin physiology that are negatively impacted by stress. A complementary clinical trial is currently done to study the efficiency of oral supplementation with our extract on stress regulation and stress related skin disorders. We believe that an IN&OUT strategy holds great potential for addressing stress-related issues and its impact on skin to break the cycle and improve overall well-being.

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