Evaluation of soothing activity of *Aphloia theiformis* extract using an innovative model of human innerved reconstructed epidermis

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Key words: human innerved reconstructed epidermis, neurogenic inflammation, soothing, ATE extract

Abstract

Sensitive skin is defined by the occurrence of unpleasant sensations such as tingling, burning, itching or pain. Many hypotheses have been proposed to explain mechanisms involved in sensitive skin. Because sensitive skin is primarily characterized by a wide variety of neuropathic-like symptoms, it is highly likely that neurosensory dysfunction in the skin represents one of the pathological mechanisms of sensitive skin. Especially, cutaneous innervation and excessive release of neuropeptides are recognized as major elements of skin physiopathology associated with neurogenic inflammation. Few experimental approaches are available to evaluate neurogenic inflammation. Most of them rely on animal models with rodent neurons.

In this study, we have developed a fully human reconstituted epidermis with iPSC-derived sensory neurons. In this model, human neurons are mature and functional. *Aphloia theiformis* extract (ATE) has demonstrated its capacity to inhibit release of CGRP (Calcitonin Gene Related Protein) upon capsaicin stimulation. Proteomics shown that ATE extract may also inhibit neurite outgrowth and regulate inflammatory response. Moreover, this active was able to stimulate keratinocyte differentiation and then can improve the skin barrier function. These findings suggest that ATE extract could be a promising active for treatment and prevention of sensitive skin prone to inflammation.

1. Introduction

Sensitive or reactive skin is a common condition that affects many people and becomes a topic of interest for cosmetics industries. According to epidemiological studies, approximately 50% of adults (60% women and 40% men) report having reactive skin, although this patient-reported prevalence varies across countries [1-3]. An official definition has been recently established by the International Forum for the Study of Itch (IFSI). Sensitive skin is now defined as "a syndrome defined by the occurrence of unpleasant sensations (stinging, burning, pain, pruritus, and tingling sensations) in response to stimuli that normally should not provoke such sensations. These unpleasant sensations cannot be explained by lesions attributable to any skin disease. The skin can appear normal or be accompanied by erythema" [3].

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Although often transient, and in many cases unaccompanied by visual dermatological responses, sensitive skin affects the quality of life [4, 5].

Sensitive skin was considered a consequence of dry skin for a long time. Because sensitive skin is primarily characterized by a wide variety of neuropathic-like symptoms, it is highly likely that neurosensory dysfunction in the skin represents one of the pathological mechanisms of sensitive skin. Neurogenic inflammation describes a mechanism by which sensory nerves contribute to inflammation. This phenomenon is mediated by the release of neuropeptides such as substance P (SP) and calcitonin gene related protein (CGRP). Since sensitive skin is induced by various environmental factors, including UV light, cold, heat and air pollution, the activation of TRP channels might represent a mechanism by which external stimuli are transferred to individuals with sensitive skin [1, 6, 7]. Among these receptors, TRPV1 is a nociceptive cationic channel responsive to high temperature (>43°C) and capsaicin is its natural agonist [8]. So, the nervous system takes part in skin homeostasis and interacts with skin cells [9].

Aphloia theiformis leaves had been traditionally used in La Reunion island to treat fevers, pain, malaria and inflammation. In the pharmacopoeia of Madagascar, the bark is also used as emetic and the leaves as antipyretic. Thanks to its anti-inflammatory properties [10, 11], ATE extract isolated from *Aphloia theiformis* leaves, could provide a new target for the treatment and prevention of sensitive skin. In this study, we used an innovative model of fully human innerved reconstructed epidermis to determine if this botanical extract is able to reduce neurogenic inflammation.

2. Materials and Methods

A specific and innovative *in vitro* fully human innerved 3D model was developed to study the effect of ATE extract on neurogenic inflammation.
For this purpose, we followed different parameters. Morphology of the RHE was evaluated thanks to hemalun-eosin and immunofluorescence stainings. The capsaicin test was used to investigate ATE extract ability to inhibit CGRP release. Proteomic analysis were carried out to better understand mechanisms involved in ATE extract efficacy.

Cell and tissue culture and treatment

The *in vitro* Reconstituted Human Epidermis (StratiCELL, RHE/001) were cultivated at the air-liquid interface during 7 days in a suitable culture medium (StratiCELL, Isnes, Belgium) within a humid atmosphere at 37°C with CO_2 5%. In parallel of RHE culture, human induced pluripotent stem cells (hiPS) were seeded in 6-well plates coated with a thin layer of BD Matrigel® and cultured at 37°C in DMEM-F12 medium supplemented with 0.1µM of retinoic acid, 1µg/mL of EPO, 10% of KSR, 1% of P/S and a cocktail of inhibitors. At day 8 the underside of the filters supporting the reconstructed epidermis were coated with a thin layer of BD Matrigel®, hiPS cells were dissociated and 140 000 cells were seeded per culture vessels. The RHE co-cultivated with human sensory neurons derived from hiPS (RHE-huSN) were maintained in culture medium with a daily refresh. The different formulations (placebo *vs* ATE 0.1%) were topically applied on the *stratum corneum* of the RHE-huSN for 24 hours (2µL). When required, the RHE-huSN were stimulated by capsaicin (10µM) in the culture medium 20 minutes before ending the experiments.

CGRP release quantification

The culture supernatants were collected and stored at -80°C until the measurements of CGRP released by ELISA (Abbexa), according to the supplier's specifications (two wells per replica, 3 replicates per condition). All treatments were compared to the capsaicin activation control condition.

Histology and immunolabeling

RHE-huSN were fixed in a 4% formaldehyde solution. For each condition, an entire sample was labeled by immunofluorescence with an anti-β-tubulin antibody to validate the presence of human sensory neurons under the membrane while two other replicates were dehydrated and paraffin embedded. Histological analysis was performed under 6μm sections for each condition stained with eosin and hematoxylin (H/E). Immunostaining on paraffin sections was performed to detect β-tubulin and neurofilament markers using specific primary antibodies and with secondary antibodies, conjugated to a fluorescent dye (Alexa Fluor 568). DAPI (4',6'-diamidino-2-phénylindole), a fluorescent molecule able to bind to DNA, was used to stain the cell nuclei. Slides were mounted with Mowiol and stored at 4°C. The capture of representative pictures/replicate was performed with a microscope Leica (DM 2000, lens 40x) combined to a camera Leica (DFC420C).

Nano-liquid chromatography and tandem mass spectrometry

Peptide digests were analyzed by Nano UPLC coupled to high resolution accurate mass mass spectrometer (Ultimate 3000 Nano RSLC ProFlow coupled to Q-Exactive Plus Both Thermo Scientific, Rockford, IL).

Overrepresentation analysis and data integration

Proteins identified by LC-MS/MS and considered as significantly regulated in relative quantitation proteomics experiment were analyzed using CORAVALID pipeline according to the method described by Hameury [12] and Ingenuity Pathway analysis (Qiagen Hidden) to draw interaction network.

3. Results and Discussion

3.1. A fully human innerved reconstituted epidermis

A new model of fully human innerved reconstructed epidermis has been developed to evaluate the efficacy of actives or formulations to reduce neurogenic inflammation.

The nervous system takes part in skin homeostasis and interacts with skin cells [9]. Skin organotypic *in vitro* systems are very interesting but are incomplete models because they lack innervation. Some *in vitro* models have been developed using re-innervated human skin explant with primary sensory neurons from the dorsal root ganglia of rats [13, 14] or a rat pheochromocytoma cell line differentiated in neuron [15]. The animal origin of neurons in these models does not seem to critically compromise their ability to interact with most human skin cells, notably because neuropeptides are highly conserved among species. However, interspecies differences cannot be ruled out. Finally, the use of animal cells has been banned in the European Union for cosmetic research and development. Therefore, the replacement of animal neurons by human neurons remains highly desirable. A recent study has shown that human iPSC-derived sensory neurons can be used in place of rodent neurons to prepare innervated tissue-engineered skin models [16]. We have also used this innovative approach to develop a fully human innervated RHE model.

Reconstituted epidermis were cultivated on inserts equipped with a porous filter with a seeding of human sensory neurons derived from hiPS on the lower face of the filter (RHE-huSN - Figure 1).

The activation of the RHE-huSN with capsaicin results in an increase of the neuropeptide CGRP release compared to the untreated condition and demonstrates that the neurons present in the model are mature and functional (Figures 2A and 2C).

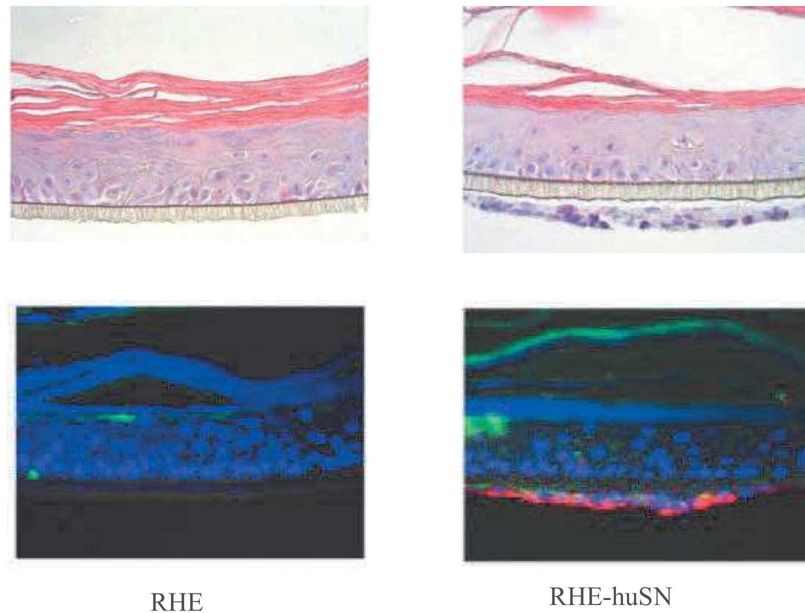


Fig.-1: Histological analysis (hemalum/eosin staining) and immuno-labelling (green: β-tubulin, blue: DAPI, red: NF-20) of RHE-huSN

Moreover proteomic analysis have shown that the presence of neurons in RHE-huSN induces significant differences in the relative abundance of 84 proteins associated with significantly enriched terms linked to the nervous system. According to our analyses, neurons seem still active in the rebuilt epidermis supplied with nerves and cells modifications, proliferation and migration could be activated. Further investigations show that neuronal development could be activated, and that cell proliferation could be regulated, limiting risks of potential anarchical proliferation. Indeed, we observed an up-regulation of neuromodulin in RHE-huSN compared to RHE. This protein plays a role in axonal and dendritic filopodia induction and is associated with nerve growth. Microtubule-associated protein 1A (MAP1) involved in neurite extension is also increased. Moreover, the up-regulation of neurofascin could translate an increase in synapse formation in the human innerved reconstructed epidermis. Furthermore, four up-regulated proteins in RHE-huSN could be involved in neurites growth: the growth factor midkine, Ras-related protein Rap-1A which plays a role in NGF (nerve growth factor)-induced neurite outgrowth, protein RUFY3 which is involved in axonal growth and neural cell adhesion molecule L1 associated with axonal growth and synaptogenesis. Dihydropyrimidinase-related protein 2 (DPYL2) is also up-regulated. This protein plays a role in neuronal development and polarity, as well as in axon growth and guidance.
Moreover, gene expression and molecules transport could be activated, showing also a higher activity in the human innerved reconstituted epidermis.

3.2. Soothing properties of ATE extract

Aphloia theiformis, of the *Aphloiaceae* family, is a tree native to the Mascarene, Madagascar and East African forests. It has a large number of medicinal uses [17] and is particularly favored for its wound-healing and antipyretic properties. The main benefit in *Aphloia theiformis* seems to lie in the richness in xanthones of its leaves demonstrating to have a positive role in a variety of physiological and disease processes including photoprotective and anti-aging properties, as well as antioxidant [18, 19], or anticancer [17].
In this context, we developed an extract from the ground leaves of *Aphloia theiformis* (ATE). This ATE extract has already demonstrated soothing effect as it is able to reduce the production of both IL-1α and

IL-8 after an inflammatory state induction in normal human keratinocytes and the production of PGE2 on a stimulated co-culture between keratinocytes and dendritic cells (data not shown).
In the present study, we have evaluated ATE extract ability to reduce cutaneous neurogenic inflammation using a fully human innervate RHE.

First of all, observations of histological hematoxylin-eosin staining revealed that every RHE-huSN sample, treated or not by ATE extract and/or capsaicin, has a morphology and cellular organization similar to RHE tissues grown in the absence of neurons. As expected, the tissues show the nuclei marked in darker blue with respect to the cytoplasm. The different cell layers of the epidermis are well observed, with a gradual differentiation of the basal layer to the formation of the *stratum corneum* passing successively through the 3 typical cell layers: the basal, spinous, and granular layers. ATE extract (0.1%) does not affect the viability and the morphology of the tissues.

The capsaicin test is a particularly relevant model since it is a selective TRPV1 agonist capable of inducing an acute nociception and neurogenic inflammation in experimental models through activation of capsaicin-sensitive peripheral afferent fibers or sensory neurons [20]. At the end of treatments with ATE extract and capsaicin stimulation, supernatants were analyzed for CGRP release. ATE extract (0.1%) appears to be able to downregulate the CGRP release induced by capsaicin stimulation by 30% (p=0.046, Figure 2B).

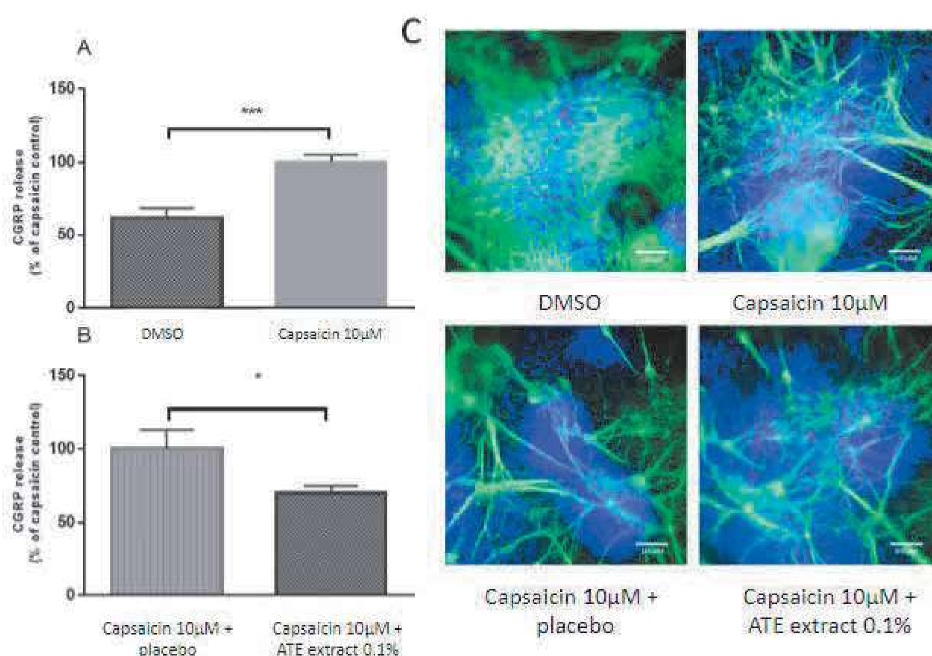


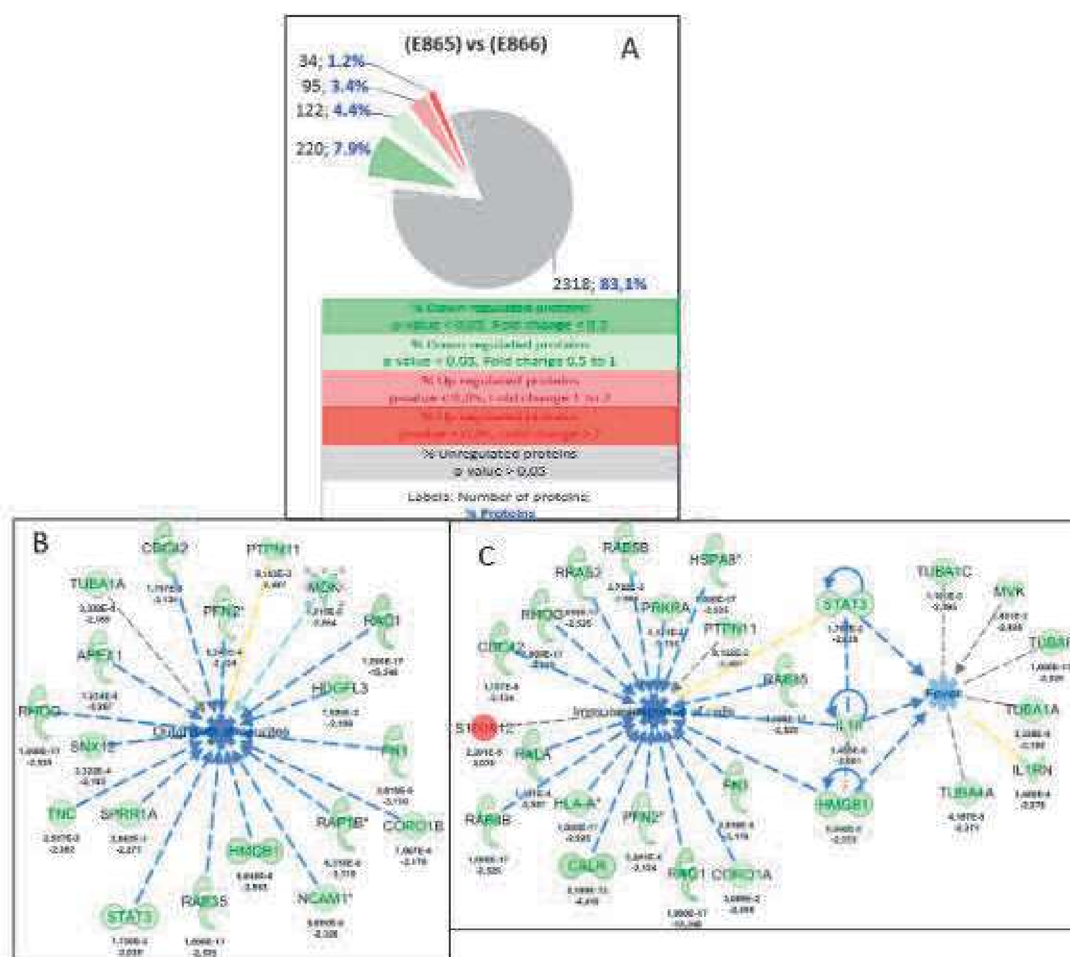
Fig.-2 : (A) CGRP released by human sensory neurons was measured on supernatants of RHE-huSN after capsaicin activation (student test - ***p<0.001). (B) CGRP released by human sensory neurons was measured on supernatants of RHE-huSN after capsaicin activation and ATE extract treatment. Results are expressed in percentage of placebo+capsaicin condition (student test - *p<0.05). (C) Immunofluorescence staining realized on human sensory neurons growing on the lower face of the membrane supporting epidermis. Staining were done after capsaicin activation (β- tubulin: green and DAPI: blue).

We further investigate the effect of this botanical extract on RHE-huSN proteome. This proteomics analysis allowed identification and relative quantification of more than 2700 proteins. Treatment of RHE-huSN tissues with ATE extract induced significant differences in the relative abundance of 254 proteins (Figure 3) Among these modulated proteins, 34 were increased by at least two-fold and 220 were decreased by at least 2-fold.

Proteomic analysis have shown that ATE extract is able to down-regulate STAT3 which acts as a regulator of inflammatory response, IL-18 and HMGB. Thus the antioxidant and anti-inflammatory properties also may, in part, account for the antinociceptive activity of ATE extract.

Moreover, ATE extract seems to inhibit neurite outgrowth according to the significant down-regulation of 19 proteins known to promote it. This can lead to a decrease in the density of innervation which, linked with the inhibition of CGRP release, leads to a decrease in the skin sensitivity. This extract also inhibits BDNF signaling pathway that can lead to a decrease of neuron dendricity and then participate to a decrease of itching.

Fig.-3 : Proteomics analysis of ATE effect on RHE-huSN. (A): Label free quantification results of ATE extract compared to control (Background t-test, *p*<0.05). (B) and (C) : IPA interacting network of outgrowth of neurites and immune response respectively.



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To summarize, this botanical extract not only permits to inhibit CGRP (a pro-inflammatory neuropeptide) release induced by capsaicin treatment but also to decrease expression of key modulators of skin innervation and neuron dendricity.

4. Conclusion

In conclusion, *Aphloia theiformis* extract is able not only to downregulate the CGRP release induced by capsaicin stimulation (TRPV1 inhibition) but also to downregulate neurites outgrowth which can lead to a decrease of skin innervation and then to a decrease of skin sensitivity. Moreover, ATE extract inhibits BDNF signaling pathway that can lead to a decrease of neuron dendricity and then to a decrease of itching. Moreover, this active regulates inflammation.
For the first time on a human model, these findings demonstrate that ATE extract can reduce neurogenic inflammation and then can be particularly relevant for topic treatment and prevention of sensitive skin.

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