Water as anti-ageing agent, thanks to osmolytes functions.

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Abstract

Osmolytes structure offer numerous free terminations –OH, and/or pairs of electrons in doublet (-O-; -S-; -N=) to stabilize proteins but also water molecules. Osmolytes family can be divided into three groups: (1) small-sized carbohydrates (trehalose, polyols, glycerol, inositol etc.); (2) amino-acids (glycine, proline, taurine etc.); (3) methylamines (glycine betaine). We focused on myoinositol and taurine.

In this study, we present results concerning a biotechnological active ingredient obtained from butterfly lavander (*Lavandula stoechas*) dedifferentiated cells. First, using full transcriptomic analysis, we demonstrated an increase of cluster genes linked to osmolytes metabolism. We demonstrated the improvement of TAUT and SMIT in parallel. Focussing on the cellular consequences and on the effects on skin. we obtained very impressive pictures of treated keratinocytes. They showed the increase of cellular surface up to 36%*** ($p<0.001$). Raman spectroscopy helped us to demonstrate the improvement of water reserves in skin explants due to the treatment. There were 69%* ($p<0.1$) more signal corresponding to water signature in the tissue. Finally, treated models showed a fully increased thickness of living epidermis but also of *stratum corneum*, a stimulated viability by 89%*** and a protection of nuclei. The importance of osmolytes in skin homeostasis was confirmed. Water management by osmolytes and by our active induced reduction of markers for stress and ageing.

Key words: hydration, osmolytes, taurine, myoinositol, ageing.

Introduction

Water is the most ubiquitous component. As universal biological solvent, it is important and essential for all living cells. Water allows chemical reactions inside cells (enzymatic reactions…) and, outside cells, a sensible balance of hydrophilic and hydrophobic interactions always controls stability of all living systems (like cells and membranes…). In human biology

as in plant biology, hydration corresponds to water absorption by the organism, improving water intake and/or reducing water loss. Different strategies exist to maintain water in the tissue, in or outside the cells. The number of potential H-bonds helps to reticulate molecules of water, thanks to osmolytes for example. Well represented in plant or human, they are small molecules composed by organic matter and soluble in intracellular media. They play a role against different environmental stress such as dehydration. In this case, lack of water could threaten cellular integrity, proteins conformation and stability. Through their structure, osmolytes offer numerous free terminations –OH, and/or pairs of electrons in doublet (-O-; -S-; -N=) to stabilize proteins but also water molecules. Osmolytes family can be divided into three groups: (1) small-sized carbohydrates (such as trehalose, polyols, glycerol, inositol etc. and their derivatives); (2) amino-acids (glycine, proline, taurine etc.); (3) methylamines (glycine betaine). We focused on myoinositol and taurine, that are really conserved and the main represented in epidermal keratinocytes [1].

Myoinositol is an isomer from the simple sugar inositol ($C_6H_{12}O_6$), presenting 6 hydroxyl terminations. It is named phytic acid in plant physiology. The sodium-dependent myoinositol transporter (SMIT) is known to be expressed by human keratinocytes under different stress conditions (osmolar stress and Ultra-Violet, UV). In another hand, taurine is a sulphur containing derivative from amino acid (NH_2-CH_2-CH_2-SO_3H). Literature indicates the presence of taurine and of its transporter (TAUT) in the epidermis and relates that the accumulation of taurine as one potential mechanism protecting epidermal keratinocytes from dehydration and oxydation [2]. Cultured human keratinocytes accumulated taurine in a concentration- and osmolarity-dependent manner. A high level of taurine protects cultures of keratinocytes from both osmotically induced and UV-induced apoptosis [3]. This so-called osmolytes strategy requires not only the expression of specific osmolytes but also the expression of their specific transporting systems. Both are important for keratinocytes capacity to maintain cell volume homeostasis in several stress conditions [1]. Ageing and especially photo-ageing is characterized by the deterioration of tissue structure and function. Reduction in keratinocyte cell size with age and downregulation of osmolytes transporters SMIT and TAUT with UV exposure were reported. This confirms that osmolytes play a critical role in cutaneous age-related alterations.

In this study we present results concerning a biotechnological active ingredient obtained from butterfly lavender (*Lavandula stoechas*) dedifferentiated cells. Using several complementary methods, we showed that this active ingredient could improve moisturizing of skin cells and of skin tissue as well, for anti-ageing benefits.

Materials and methods

Plant material and cells multiplication:

In vitro cultures were established from leaves of *Lavandula stoechas* obtained from the Lepage nursery company (France). Leaves surface was sterilized using 70% of ethanol during 30s, followed by 3% of sodium hypochlorite during 8min and, finally, rinsed with distilled water 3 times. Then, leaves segments were disposed on gelose Murashige and Skoog (MS) (complemented with vitamins; sucrose ; 1-Naphthalene acetic acid (NAA); 6-Benzylaminopurine (6BAP); 5g/L agar), kept in dark and 22°C. After one month, we obtained callus culture that we maintain monthly in the same conditions. The suspension step was initiated in Erlenmeyer in MS with vitamins, sucrose, NAA and 6BAP and supplemented with hydrodistilated *Lavandula stoechas* extract and marine spring water. After pH adjustement and autoclaving, the cells were kept in suspension, in dark and 22°C and maintained every week.
The scale up for biomass production was progressively realised with Erlenmeyer of 0.2L, to homemade bioreactor of 2L-10L. After 10-20 days of culture, the biomass was collected by filtration with nylon filter of 50µm, rinsed, and then frozen

Obtaining of the extract:

The extract was obtained using propane-1-3-diol on cells ground with high-pressure homogenizer. It was described as cell pulp for its viscous turbid liquid aspect with some sedimented particles under resting condition. The color is from light brown to dark brown.
We also characterised our extract with a minimum of 0.4% of dry matter, less than 1% of mineral content, more than 25ppm of glutamic acid (UPLC acquity H class from waters, compared to a standard range with (L)-glutamic acid). The vitamin B6 content is 0.05ppm (liquid chromatography with a fluorescence detector according to European standard EN 14164:2014).

Biological material:

2D cultures : All the experiments were performed on normal human keratinocytes cultivated in monolayer conditions (used in passage 2 to 3).
3D cultures in reconstructed epidermis according to our knowledge. . Third passage, normal, human, epidermal keratinocytes were seeded over two days in 12 mm diameter inserts with complete medium (complete EPI Life) at 37°C and 5% of CO2. At baseline the cells were induced to differentiate with the passage of Air-Liquide interface to form a multi-strata epidermis. Further to 18 days of incubation, some models were pre-treated with the active

substance for 24 hours. Then, at Day 19, tghey were stressed for 24 hours at 400 mosm before being set in formaldehyde for the preparation of histological sections.
Some experiments were also realised on explants models maintained in survival conditions (diameter: 12±1 mm, collected after an abdominoplasty operation).

Histological analysis:

The histological sections were coloured with hematein-eosin (H.E.). These two colorants combine hematein, which colours the nucleus in purple, and eosin, which colours the cytoplasms in pink. The colorations heightened contrasts thus enabling the number of pyknotic cells to be counted. Analysis and counting were performed with a NIKON Ti-U microscope and NIS elements software (NIKON). For complementary statistical analysis, raw data were transferred and processed using Microsoft Excel® software. Intergroup comparisons were performed using Student's test. Results were considered significant at the 5% threshold. The results of the statistical tests were marked as follows: * when $p<0.05$; ** when $p<0.01$ et *** when $p<0.001$.

Full transcriptomic analysis:

The cells were cultivated over 3 to 5 days on a plastic support in the presence of complete growing environment, at 37°C, 5% CO2 and saturated humidity. The Lavandula stoechas extract was added (0.05% in culture medium) on cultures presenting 70% to 80% confluency, during the last 24 hours of culture). We analysed gene expression using the whole transcriptome method following the manufacturer's instructions : RNA extraction using Qiagen's RNeasy Plus Mini ® kit, amplification and labelling using Agilent's Low input Quick Amp Labeling® kit, Hybridisation of fragmented RNAc on the SurePrint G3 Human Gene Exp V3 chip (approx. 60,000 probes) performed on the Agilent hybridisation station, images of each SurePrint G3 Human Gene Exp V3 probe acquired using Aglient's SureScan DX Microarray Scanner. We used R statistical language and R Studio software for fold change analysis.

RT-qPCR:

The cells were cultivated over 3 to 5 days on a plastic support in the presence of complete growing environment, at 37°C, 5% CO2 and saturated humidity. The Lavandula stoechas extract was added (0.05% in culture medium) on cultures presenting 70% to 80% confluency, during the last 24 hours of culture). We analysed gene expression using the qPCR performed following the manufacturer's instructions and this various steps: RNA extraction using

Qiagen's RNeasy Plus Mini ® kit, conversion of RNAm to DNAc using Invitrogen's Superscritp IV, real time DNAc amplification using Applied Biosystems's PowerUp SYBR Green Master Mix kit and primer couples to genes of interest, real time amplification is performed and monitored on Applied Biosystems's QuantStudio 3. We considered fold changes using Microsoft's Excel software.

ELISA test:

Keratinocytes were seeded in 100 mm Petri dishes for 3 or 4 days in complete KSFM medium. (Keratinocyte Serum Free Medium, Gibco). Then cells were pretreated with or without the Lavandula stoechas extract (0.05% in culture medium) for 24h and stressed with hyperosmolar stess (400 mosm/g /L) for 1 night. Fresh medium was added for 2h and cells were trypsinized and counted. Then, the cell pellets are dry frozen at -80°C.

For the ELISA analysis, the cell pellets were thawed and taken up in the assay buffer, sonicated and refrozen until freeze-drying. The lyophilisates were taken up in 1 ml of assay buffer and centrifuged. Quantification of taurine was performed using a taurine assay kit (Cell Biolabs, ref. MET- 5071) and the reading acquired using the software Fluostar OMEGA.

Statistical analysis:

Raw data were transferred and processed using the Excel software. The test used was the Student t test on paired data sets. Outliers were eliminated by the Grubbs test (statistical test used to detect outliers). A difference between two groups was considered as statistically significant at a significance level of 10% (#$p<0.1$). If the p-value was less than 0.05, less than 0.01, less than 0.001and less than 0.0001 *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$, respectively, were noted.

Cellular morphological stability analysis:

Cells were cultivated for 3 days in a 24-well plate in complete KSFM culture medium. Cell seeding was performed in line with donor age and the number of passes, to obtain, at the end of the experiment, cultures presenting with the same confluency percentage (70%-80%). Cells were then pre-treated with or without the Lavandula stoechas extract (0.05% in culture medium) over 24 hours. Afterwards they underwent 16-hours osmotic stress and then were treated again with or without the active over 2 hours. At the end of this 2-hours period, the cells were photographed (with a NIKON Ti-U microscope) and then dry frozen at -80°C for later

analysis. We used the Nikon NIS-Elements Ar software for automatically analyse cell surface area. We purchased statistical analysis as well.

RAMAN spectroscopy:

The explants were placed in survival in medium (BIO-EC's Explants Medium) into incubators. On Day0, Day1, Day2 and Day 5 Lavandula stoechas extract (0.5%) was applied topically with 2 µl per explant (2 mg/cm2). On Day0 three explants of the T0 batches were sampled and sectioned in two. One part was fixed in a buffered formol solution and the other was frozen at –80°C. On Day6 three explants from each batch (T and A) were sampled and processed in the same manner as on Day0.

After 24 hours in the buffered formol, the samples were dried, immersed in paraffin and then sectioned using a microtome or a cryomicrotome (5 µm, Leica RM 2125 or 7µm, Leica CM 3050, respectively). Afterwards the sections were mounted on silane-coated and non-silane-coated histological glass slides (Superfrost®) and then microscopic observations are performed in optic microscopy using a microscope (Leica type DMLB or Olympus BX43) and the images and analysis are obtained using a camera (Olympus DP72) and the Cell^D software.

Epidermal intracellular water is measurable by analysis of tyrosine conformation (moisturised form/non-moisturised from ratio) using Raman spectroscopy. The Raman spectra were obtained with a confocal Raman Xplora spectrometer (Horiba, Jobin Yvon) using a 532 nm laser source. For each explant, two frozen, 7 µm sections were placed on CaF2 slides. A long-focus lens (PL Fluotar L 100_/NA 0.75 WD 4.7) was used to focus the laser on the surface of the samples and to collect the dispersed light. The collected light was then filtered using a notch filter and a 1200T dispersion network, all providing 10 cm-1 spatial resolution. The confocal gap was fixed at 300 µm and the slit at 100 µm. Acquisition time was 30*20 seconds with 20 accumulations per point. The Raman signal was recorded with a CDD camera and spectral acquisition was performed with LabSpec 6 (Horiba Scientific) software. Each spectrum was adjusted and then the baseline was corrected using an automatic polynomial. The Raman spectra were standardised over the entire spectral range. The epidermis moisturising level was evaluated using the ratio between the air present under the peak at 850 cm-1 and that at 830 cm-1. 6 points per explant, i.e., 18 points per batch, were analysed on living epidermis.

Student's t-test gives the probability that two batches be significantly different. The difference between two batches is significant if $p<0.1$ (#; limit of significance), i.e., a 90% probability for two batches to be significantly different or $p<0.05$(*), i.e., a 95% probability for two batches to

be significantly different or p<0.01(**), i.e., a 99% probability for two batches to be significantly different.

Results

EXPRESSION OF GENES IMPLICATED IN CELLULAR HOMEOSTASIS AND OSMOLYTES METABOLISM

In the presence of *Lavandula stoechas* extract, used at 0.05%, molecular analysis demonstrated increases of expression of 3 enzymes implicated in taurine metabolism: BAAT of +75% (Bile Acid-CoA: Amino Acid N-Acyltransferase), CDO1 of +94% (Cysteine Dioxygenase Type 1) and GADL1 of +125% (Glutamate Decarboxylase Like 1). In addition, the taurine receptor (TAUT) was also increased by +51% in presence of the active substance compared to the untreated cells (detection by qPCR).

As shown in figure 1 below, pretreatment with the *Lavandula stoechas* extract (at 0.05%) increased the level of taurine on three different donors respectively by +27% (#), +42% (*) and +24% (#) after stress. Statistical analysis indicated that mean of our experiments showed a significant induction of taurine protein as well.

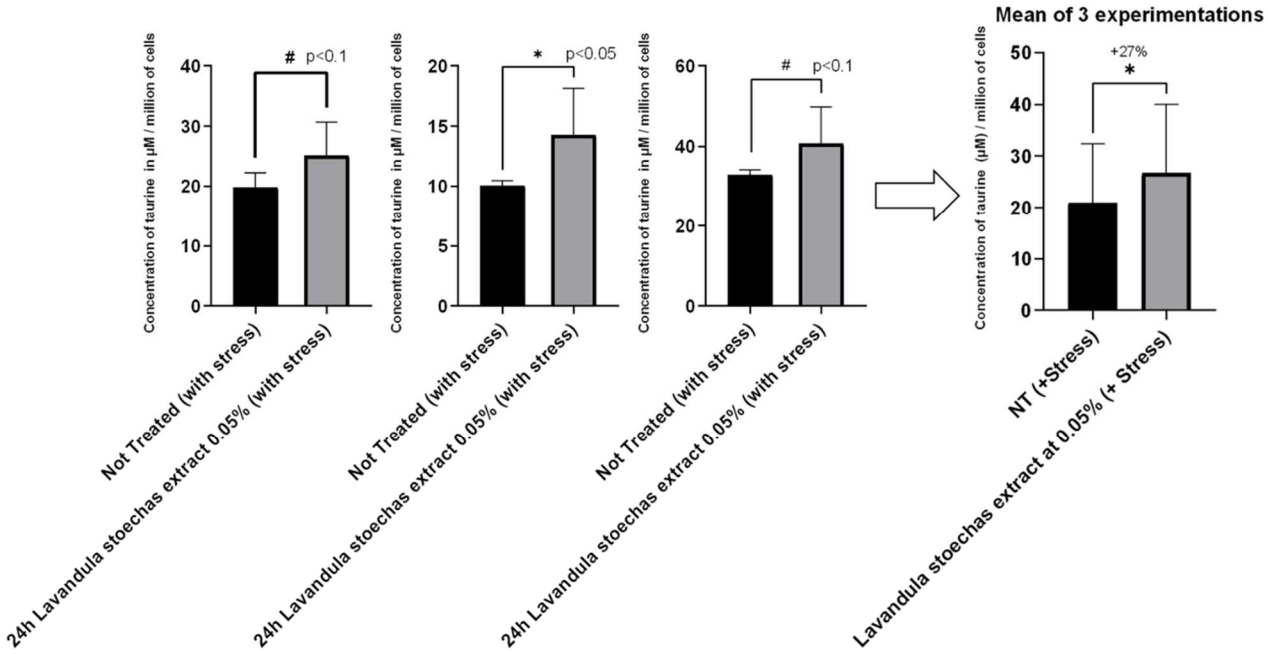

Figure 1: ELISA quantitation of taurine in NHEK cultures after 400 mosm stress (+/- the *Lavandula stoechas* extract), 3 different experiments on different donors and mean of experiments.

As shown in the graph below (figure 2), the *Lavandula stoechas* extract increased SMIT and TAUT genes expression by 21% and 40% respectively after 24 hours of pretreatment and one night of hyperosmolar stress.

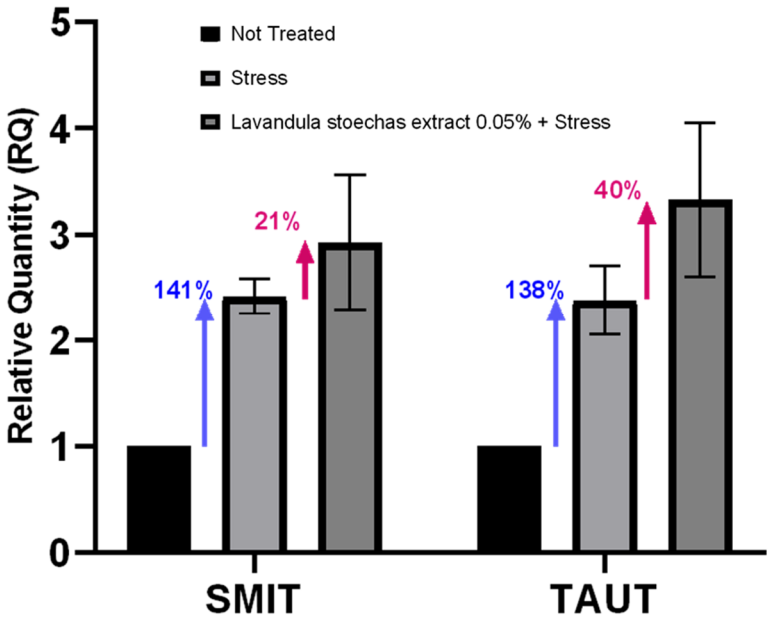

Figure 2: SMIT and TAUT gene expression after 24 hours with the *Lavandula stoechas* extract treatment and 1 night of hyperosmolar stress on NHEK

MORPHOLOGICAL PROTECTION

Exploring a higher level of action, we focused on cellular scale before the tissue level. Morphological observations helped us to describe the effect of the active. We analysed and compared the cell size and shape in different conditions.

As shown in figures 3 and 4, Compared analysis of cell size confirmed that, under stress conditions, mean cell surface area significantly decreased by -42% ($p=4.08.10^{-8}$) compared to unstressed, untreated cells, i.e., analysed, mean size decreased from 872+/-165 to 509+/-151. If the cells were pre-treated with the *Lavandula stoechas* extract used at 0.05% for 24h, they were larger than the stressed cells and their mean surface area increased from 509+/-151 to 691+/-155, i.e., a significant increase of +36%***($p=0.00014$) with a 50% protection compared to the stressed, untreated cells.

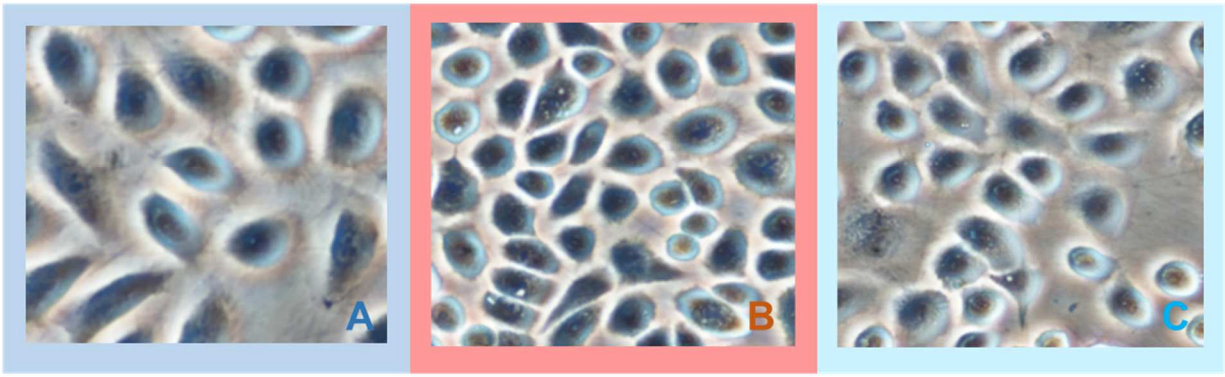

Figure 3: Microscopic observation of the surface area of epidermal keratinocytes after 24 hours of the different treatments: A-Unstressed, untreated cells. B-stressed, untreated cells. C-stressed cells, pre-treated with the *Lavandula stoechas* extract used at 0.05%.

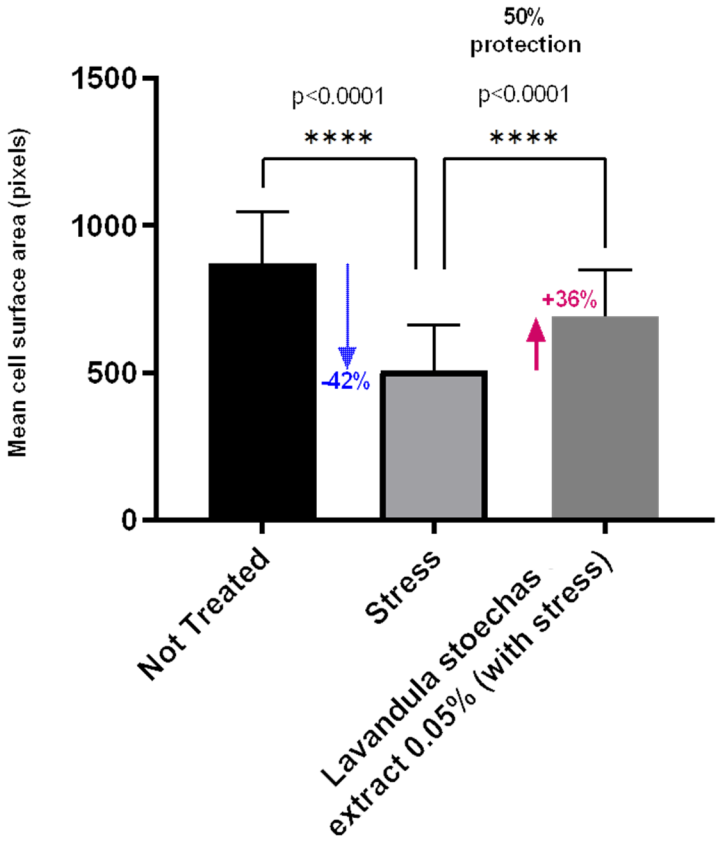

Figure 4: Analysis of mean cell surface of human epidermal keratinocytes under stress conditions and pre-treated with the *Lavandula stoechas* extract used at 0.05% compared with untreated, stressed cells.

In addition and at the tissue level, intracellular water can be measured by analysis of tyrosine conformation with Raman spectroscopy. It is a non-destructive investigation method based on the detection of photons dispersed inelastically further to interaction between a sample and a

laser source. Several parameters can be characterised on the skin by Raman spectroscopy: moisturising, stratum corneum lipid conformation or the synthesis of molecules linked to NMF formation [4].

In the present study, epidermis moisturising was evaluated by analysing tyrosine conformation (Fermi resonance). In its well-moisturised form, tyrosine presents a characteristic peak at 850 cm^{-1} in Raman spectroscopy whereas the characteristic of its unmoisturised form is the presence of an 830 cm^{-1} peak [5]; see figure 5. Large values of the ratio between the intensities at 850 cm^{-1}/830 cm^{-1} indicated sufficient skin tissue moisturising.

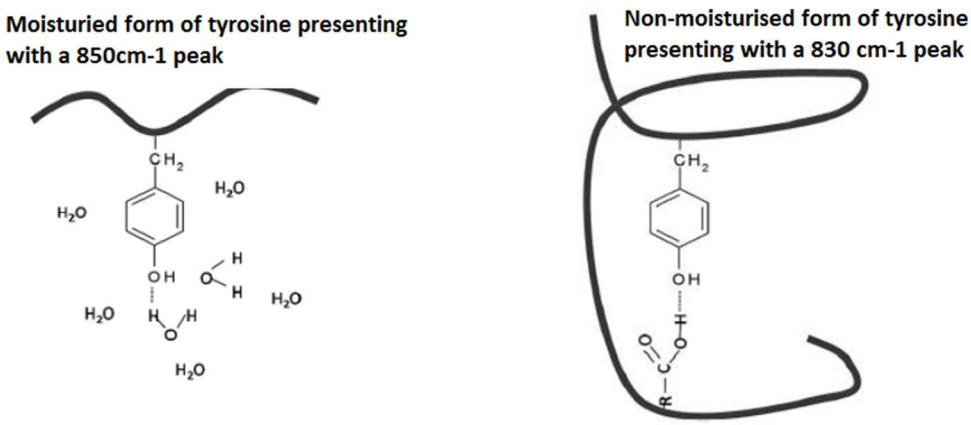

Figure 5: Tyrosine conformation in Raman spectroscopy.

At Day6, in the explants treated with the *Lavandula stoechas* extract used at 0.5%, the moisturising activity presented a significant increase of +69%#. That corresponds to the tyrosine's "moisturised form" to its unmoisturised form" ratio, that was 4.79 (I 850/I 830 cm^{-1}) compared to the reference batch (R) whose ratio I 850/ I 830 cm^{-1} was 2.83. The results are given in figure 6.

	I 850 / I 830 cm-^{1} (U.A)		
	R0	R J6	A J6
Mean	1.18	2.83	4.79
Standard deviation	0.70	2.15	3.86

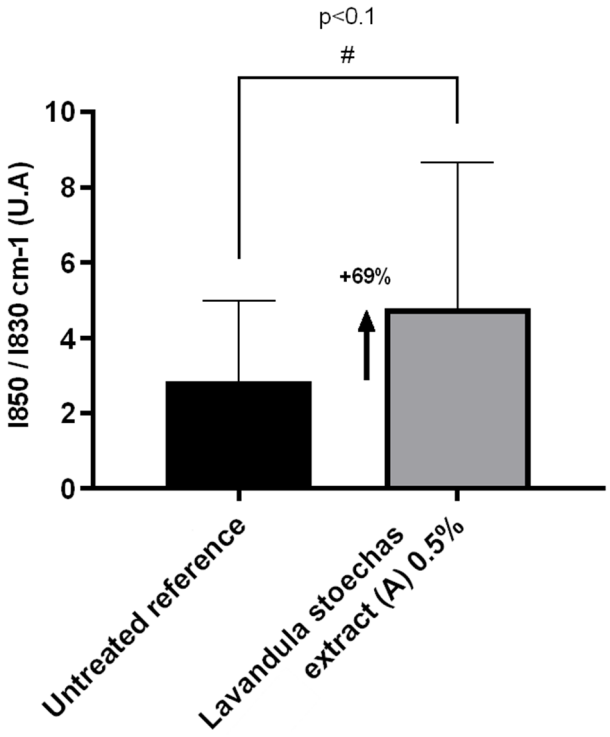

Figure 6: Comparison of Ratio I 850 / I 830 cm^{-1} between skin explants treated with the *Lavandula extract* (A) used at 0.5% compared to the untreated reference (R) at Day6**.**

To continue with 3D models, we analysed morphological organisation of a reconstructed epidermis treated with the *Lavandula stoechas* extract (used at 0.5% in topical application and 0.05% in systemic treatment) even in osmolar stress conditions.

Stress conditions induced the presence of pyknotic cells in the reconstructed, human epidermis (RHE) compared to the unstressed control. The reconstructed models pre-treated for 24 hours with the Lavandula stoechas extract and then stressed exhibited a lower number of pyknotic cells compared to the untreated, stressed models (figure 7).

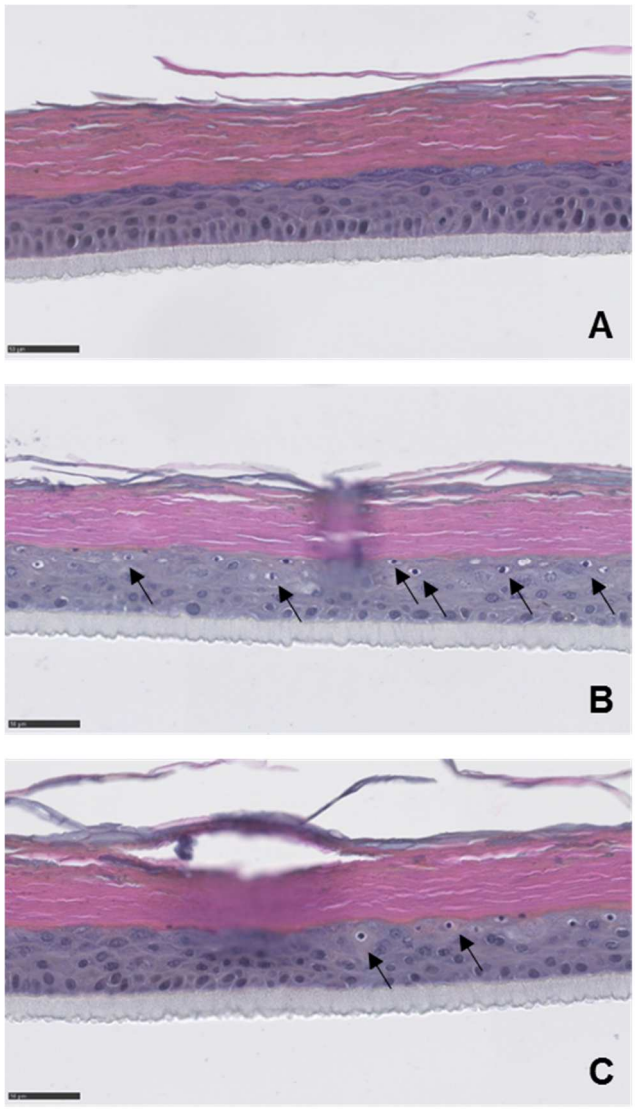

Figure 7: Microscopic observations of pyknotic cells (arrows)
in reconstructed human epidermis in different conditions:
A-unstressed, untreated. B-stressed, untreated.
C-stressed, pre-treated with the *Lavandula stoechas* extract (scale bar indicates 50µm).

In the RHEs under stress conditions the number of pyknotic cells increased significantly by +89% *** ($p=3.9.10^{-7}$) compared to untreated 3D models. Altered cells are more numerous, they increased from 2.3 per mm of epidermis for untreated conditions to 20.8 per mm of epidermis in stressed conditions.

With pre-treatment for 24 hours with the *Lavandula stoechas* extract, the number of altered cells decreased significantly by -38%** ($p=0.0037$) compared to stressed control; as the number of pyknotic cells decreased from 20.8 to 12.9 per mm of epidermis, i.e., 43% protection compared with stressed, untreated models (Figure 8).

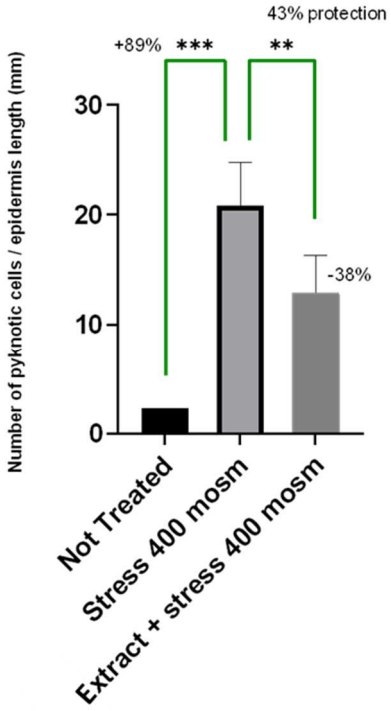

Figure 8: Analysis of the number of pyknotic cells in the reconstructed human epidermis in different conditions (untreated, stressed and untreated or pre-treated with the *Lavandula stoechas* extract and then stressed (Experiment n=6).

The last figure 9 illustrate a trend of a better organisation of the living epidermis and an also an increase of the whole epidermis thickness (from 94 µm to 124µm) due to the treatment with our extract. This confirm that there is a real effect on metabolism and/or differentiation of keratinocytes in stressed conditions but also in normal once, going further than hydration.

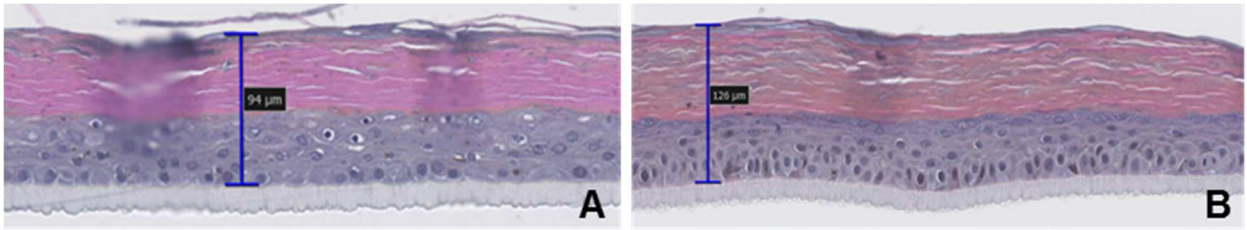

Figure 9: Measurement of reconstructed epidermis thickness in control condition (A) and when treated with the *Lavandula stoechas* extract (B)

Discussion

In this study we obtained results concerning a biotechnological active ingredient obtained from butterfly lavander (*Lavandula stoechas*) dedifferentiated cells. In another hand, it is a real occasion to discuss about potential links between molecular hydration (that could be considered as basic) and anti-ageing properties.

First, using full transcriptomic and PCR analysis after 24h of contact on human keratinocytes, we demonstrated an increase of cluster genes linked to osmolytes metabolism. Our extract stimulated BAAT, CDO1, GADL1 genes that are coding for taurine precursors. It increased the transcription of taurine but also the expression of the corresponding protein by 27% (ELISA method), even in osmotic stress conditions. Using RT-qPCR, we demonstrated the improvement of TAUT and SMIT (osmolytes transporters) in parallel [2]. The extract enhanced numerous genes involved in expression and transport of taurine. It also improved aquaporins (AQP) genes in keratinocytes cultures (data not shown). Thus, in a first point of view, the *Lavandula stoechas* extract could be presented as an active ingredient for hydration. Literature on osmolytes and taurine evoke also a decrease of synthesis and transport with ageing and environmental stress.

Secondly, we wanted assess the efficacy of our extract on 3D models, to access to consequences of osmolytes induction, closer to vivo condition. We obtained very impressive pictures of treated keratinocytes. After their analysis, we measured increases of cellular surface up to 36%*** ($p<0.001$) in condition of treatment. If keratinocytes were put in dehydrated conditions, water lost corresponded to 42%*** and if they were also treated by the extract the loss of water was reduced by 50%, to reach only 21% of loss. The treatment with the extract induced a modification of cell surface and shape thanks to water retention and osmolytes function as described [1]. The same trend was observed in skin explants or reconstructed epidermis. Raman spectroscopy showed an improvement of water retention on the tissue with +69%* ($p<0.1$) of signal corresponding to water signature. While reconstructed epidermis submitted to osmotic stress and treated with the extract exhibited a fully increased thickness of living epidermis but also of *stratum corneum*, a stimulated viability by 89%*** and an alteration of nuclei (pyknotic cells) reduced by 38%** ($p<0.01$). Those results indicates that there are not only water retention but also an improvement of keratinocytes metabolism and differentiation as well, to provide a better barrier function, even in stress conditions. This is definitely a sign of anti-ageing properties, as indicated by bibliography [6].

Taking the example of the development of our extract and its cosmetics properties, we demonstrated once again the importance of water management in skin cells biology, thanks to osmolytes function. Water is held surrounding cells to be available for all functions for viability, morphology, differentiation and protection, even in stress conditions exacerbating ageing dysfunctions.

Conclusion

In this study, we confirmed the importance of osmolytes in skin biology and homeostasis. Water management by osmolytes induced reduction of markers for stress and ageing. That's why water and its retention into cells or skin tissue could be considered as the best anti-ageing active. The extract that we developed from *Lavandula stoechas* stem cells acts on water management by osmolytes and particularly by taurine to induce anti-ageing properties.

Acknowledgments : none

Conflict of interest: none

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