SYNERGIE SKIN
CLEAN SCIENCE

CLINICAL DATA – MARJORAM LEAF EXTRACT

Cosmetic uses
Properties:

- Revitalises fibroblasts architecture, galvanises cell tonicity, fibroblasts are awakened, and their optimum activity restored
- Reactivates gene expressions and awakens cell's epigenetic pattern acting through the inhibition of DNA methylation.
- Restores the optimum fibroblast machinery for an improved neo-collagen network.

Applications:

- Increase skin firmness and density
- Anti-ageing skin care

Fibroblast: A powerful machinery
Metabolic and mechanical functionality of fibroblasts to correct skin ageing: in a nutshell.

The skin beauty is characterised by an internal complexed organisation which is principally exercised by the fibroblasts in the dermis. Indeed, fibroblasts are powerful production units which continuously produce the components of the extracellular matrix, assuring its perpetual renewal. Healthy fibroblasts mean a healthy skin. Two functionalities of the fibroblasts contribute to the good health of the fibroblasts:

The metabolic functionality which is characterised by the ability of the fibroblasts to express the genes and consequently the proteins (including collagens) essential for their healthy life and their good cellular environment.

The mechanical functionality which is characterised by the presence of an efficient cytoskeleton including actin fibres. These actin fibres assure internal traction forces in the cells which allow the mobility of the cells, their contraction, and attachment to the matrix and also to the induction of the essential gene expression which fibroblasts need.

Healthy young fibroblasts, which are the cells of our interest, possess the right molecular machinery to correctly express the useful genes which are at the end translated into functional proteins. Everything is partly controlled by a functional epigenetic mechanism (metabolic properties) and by internal tensions thanks to an organised cytoskeleton (mechanical properties). The latter mostly comprises of actin and myosin contributing to cellular traction forces on the extracellular matrix (ECM), to which fibroblasts adhere.

These traction forces also serve other important morphogenetic functions, in particular the reorganisation and alignment of collagen to form relatively large-scale anatomical structure. The organised cytoskeleton is responsible of external tension of the cells with

the ECM. As a consequence, the cells in a good shape are able to synthesise and secrete the right amount of proteins necessary to the ECM organisation and allow a strong adherence of the cells to the ECM (Figure 1).

One of the main impacts of ageing on human skin are its changes of mechanical properties. The majority of studies attribute these changes predominantly to the altered collagen and elastin organisation and density of the ECM. However, individual dermal fibroblasts also exhibit a significant increase in stiffness during ageing *in vivo*, a mechanism that may contribute to the age-related impairment of elastic and firmness properties in human skin. The altered mechanical behaviour might influence cell functions involving the cytoskeleton, such as contractility, motility and proliferation, which are essential for the reorganisation of the ECM. Decrease of protein synthesis and secretion are a consequence of these altered cell functions, the cells are in a resting state. These linked mechanisms are dependent on environment which can be addressed with epigenetics (Figure 1).

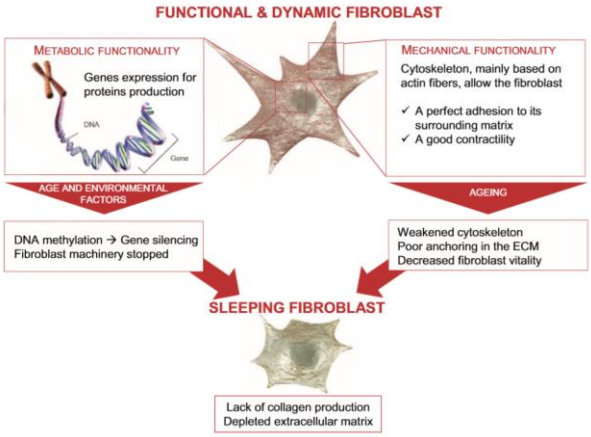


Figure 1 - Metabolic and mechanical functionalities of the healthy fibroblasts versus the aged and environmental impacted fibroblasts.

Collagen network and ageing

Dermal collagen fibre bundles are the major structural elements in the dermis and are responsible for skin firmness. The dermis contains predominantly type I collagen. Reduced synthesis of collagen is characteristic of chronologically aged skin. As a consequence, 3D organisation and functionality are progressively deteriorated. Dermis thickness is decreased by 2—80% during ageing process. The classical anti-ageing approach consists of stimulating the quantity of type I pro-collagen. However, formation of collagen fibres in skin is much more complex and is not only restricted to collagen type. Collagen-degrading matrix metalloproteinases (MMPs), which are responsible for producing collagen fragmentation, are gradually up-regulated in chronological ageing[1]. Elastin, a component of elastic fibres, is also broken down during skin ageing.

At first, a collagen precursor form is synthesised and secreted outside the cell where the propeptides will be processed by specific endoproteases, such as procollagen C-proteinase (PCP) or bon morphogenetic protein-1 (BMP-1), to form the fibrils. In the skin this proteinase is processing mainly collagen I, III and V. Fibres then need to be

organised as heterotypic fibrils composed of collagen type I and V, and finally cross-linked by lysyl-oxidase (LOX and LOXL1), also secreted as a proform processed by BMP-1. If one of these steps is lacking, as evidenced by knocking down experiments or pathologies, collagen fibre diameter is non-homogeneous, fibrils are not properly aligned in fibres and, as a consequence, collagen network loses its mechanical strength.

Cell cytoskeleton and mechanical properties linked to gene expression and protein synthesis – The ageing impact.

Cells mechanical properties and the state of the cytoskeleton affect the interaction between fibroblasts and the ECM. The crawling locomotion of fibroblasts results from the exertion of contractile 'traction' forces on ECM environment. Considerable evidence shows that these traction forces also serve other important morphogenetic functions, in particular the reorganisation and alignment of collagen to form relatively large-scale anatomical structures. These cellular traction forces are known to be generated by networks of cytoplasmic actin and myosin. Actin is involved in both stretch and adhesion fibres. Cell motility and proliferation could be affected by cytoskeletal changes.

In recent years, it has become increasingly evident that the cytoskeleton and its mechanical state are key elements for mechanosensitivity and mechanotransduction[2, 3], which play an essential role in cellular regulation for differentiation, proliferation[4], and gene expression[5]. Indeed, Lambert *et al.*[6] demonstrated that the synthesis of collagen and other extracellular matrix proteins is regulated by mechanical forces when comparing expression patterns of skin fibroblasts in stressed and relaxed collagen gels[7].

A possible link exists between age-related changes in cell proliferation and the observed higher cell stiffness in connection with change in actin organisation. Ageing leads to a high degree of actin polymerisation[8], and decreased growth rate, as well as the replicative lifespan of a fibroblast culture[9]. One study clarified how age-related changes in the mechanical properties of cells influence the expression of ECM proteins[8]. Mechanical changes may reveal new aspects of the age-related degradation of the elastic properties of the dermis therefore of the skin firmness. In fact, cellular stiffness is significantly increased in dermal fibroblasts during ageing *in vivo* [8]. Age-related changes have been observed for protein synthesis and production of ECM components. Besides this direct effect on the organisation of the ECM, changes of the actin cytoskeleton could contribute to the altered gene expression in aged fibroblasts, leading to a reduced synthesis of ECM proteins.

Fibroblasts play a crucial role in dermis remodelling. In young healthy skin, dermal fibroblasts attach to intact collagen fibrils through numerous focal adhesions and exert traction forces to achieve dynamic mechanical tension. Such tensions induce morphological changes to more stretched fibroblasts and stimulate production of ECM components including collagen, matrix metalloproteinase (MMPs) growth factors and cytokines.

In chronological ageing skin, fibroblasts functions and morphology are altered, and dermis remodelling is impaired. This is partly due to the increase of MMP-1 expression that contributes to collagen fibrils fragmentation. The loss of ECM affects fibroblast

adhesion which decreases their mechanical stimulations leading to cell collapsing, low levels of collagen production and high levels of collagen-degrading enzymes expression. In addition, fibroblasts attached to the ECM induces changes in actin cytoskeleton and fibroblasts contractility. Collagen and hyaluronic acid fillers are extensively used to temporarily improve skin appearance and firmness by restoring loss of mechanical stimulation to fibroblasts, however, other anti-ageing treatment alternatives such as soluble compounds could be tested for their ability to stimulate dermal fibroblasts contractility to restore dermis ECM integrity.

DNA methylation, an epigenetic mark

Ageing can be described as a slow, time-dependent decline of a set of multiple biological functions. In some biological pathways, functional decline can be defined in a mono-causal way, such as the decline of resting metabolism, whereas in other pathways the scope of the decline is rather broad and elusive, such as for reduced stability of epigenetic patterns. Although epigenetic patterns change dramatically during development, these early events are biologically programmed and necessary, whereas alterations of the epigenome in adult somatic tissue may reflect ageing-associated events from environmental cues.

Epigenetic mechanisms regulate the interpretation of genetic information and thus have the ability to produce different phenotypes from a single genotype. It's very understandable with genetic twins, who are not phenotypically identical because of different environmental conditions. Indeed, epigenetic mechanisms are generally considered to represent a regulatory interface between environmental cues and the genome. As people age, drastic changes occur in their DNA methylation patterns, which are though to act as a 'second code' on top of the DNA that can lock genes in the on or off position, (genes are silenced in the off position).

The only epigenetic modification of mammalian genomic DNA is methylation of the cytosine (C) residue at the 5-position resulting in the formation of 5-methylcytosine (m5C). Inserting methyl groups in the DNA promoter changes the appearance and the structure of DNA, modifying gene's interactions with the transcription machinery. DNMTs (DNA methyl transferases) are methylases which are responsible for methylation of DNA promoter, which switches off the expression of the genes (Figure 2). DNMT1 have maintenance methylation function, inherited, steady state, structural epigenetic pattern. DNMT3a have de novo methylation function which acquires environmentally driven epigenetic changes.

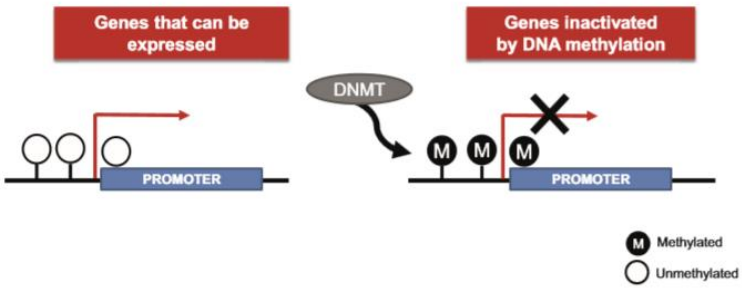


Figure 2 - DNMTs, responsible for methylation of DNA promoter which switch off the gene expression.

The process of ageing results in a host of changes at the cellular and molecular levels, which include senescence, telomere shortening, and changes in gene expression. Epigenetic patterns also change over the lifespan, suggesting that epigenetic changes may constitute an important component of the ageing process and become a hallmark of ageing. The age-specific draft in DNA methylation can be divided into global hypomethylation and local hyper methylation.

Marjoram leaf extract – A waking up solution for tired cells.
Safety/Tolerability of the product
Marjoram leaf extract was tested to ensure its safety in the recommended condition of use. Marjoram leaf extract doesn't irritate the eyes and skin under conditions of use. No indication of skin sensitisation was observed.

Description of the plant
Marjoram is a somewhat cold-sensitive perennial herb or undershrub with sweet pine and citrus flavours. Leaves are smooth, simple, petiolated, ovate to oblong-ovate, (0.5-1.5cm) long, (0.2-0.8cm) wide, with obtuse apex, entire margin, symmetrical but tapering base, and reticulate venation. The texture is extremely smooth due to the presence of numerous hairs.

Origanum majorana is a perennial small plant that grows rapidly. An aromatic plant, it measures 60cm high. The leaves are coloured between grey and green and are simple and opposed. The flowers are assembled in globular and, axillary ear are zygomorphes and coloured in white and pink. As all Lamiaceae, the stems possess a square section. The blossoming occurs between June and September. The pollination is entomogam which means realised by insects. Next, the multiplication occurs in the nature by dissemination of the seeds or by division. Its environment is diversified: mountainous area, stony, limestone and dry grounds, and with a sunny exposure.

Distribution:
Marjoram is originated from the Mediterranean area. It is cultivated today all around the world. Marjoram leaf extract is manufactured from organic certified Marjoram cultivated in Egypt or Spain.

Uses:
Marjoram was known to the Greeks and Romans as a symbol of happiness. Leaves have been reported to improve digestion and relieve digestive problems as well as disorders related to the respiratory tract. Marjoram is mostly used for seasoning soups, stews, dressings, and sauce.

Marjoram leaf extract: Processing, Composition and phytochemistry
Marjoram leaf extract is an aqueous extract of *Origanum majorana* leaves. The extraction process is optimised to obtain a preservative-free powder standardised on the sum of two specific phytochemical tracers: The C-glycoside flavonoid Vicenin-2 and O-glycoside flavonoid Luteolin-7-O-Glucuronide. An example of typical phytochemical composition of the plant extract matter is represented in Figure 3.

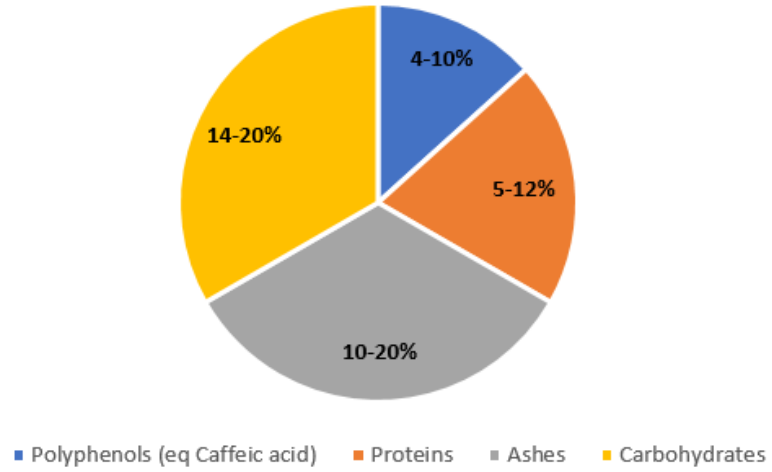


Figure 3 Typical phytochemical composition of the plant extract matter (based on 3 representative batches).

A Demonstrated efficacy

Galvanising cell-matrix interactions

Marjoram leaf extract reinforces cell structures, and increases cell tension to awaken and re-activate skin cells in order to synthesise collagen and partners in the ECM. Marjoram leaf extract restores mechanical and metabolic properties of the fibroblasts in order to induce collagen synthesis and functionalisation to get a fully filled extracellular matrix (Figure 4).

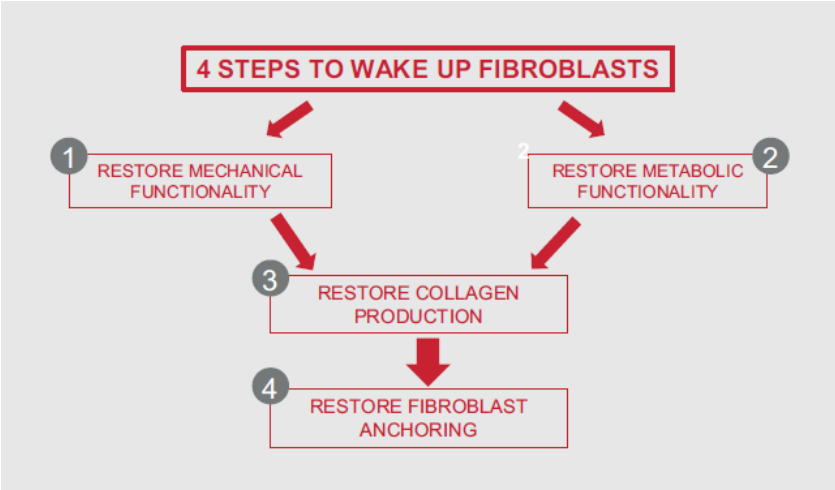


Figure 4 - Marjoram leaf extract acts on 4 steps to wake up fibroblasts.

Efficacy

Reinforce fibroblast intracellular structures and contractility

Objective

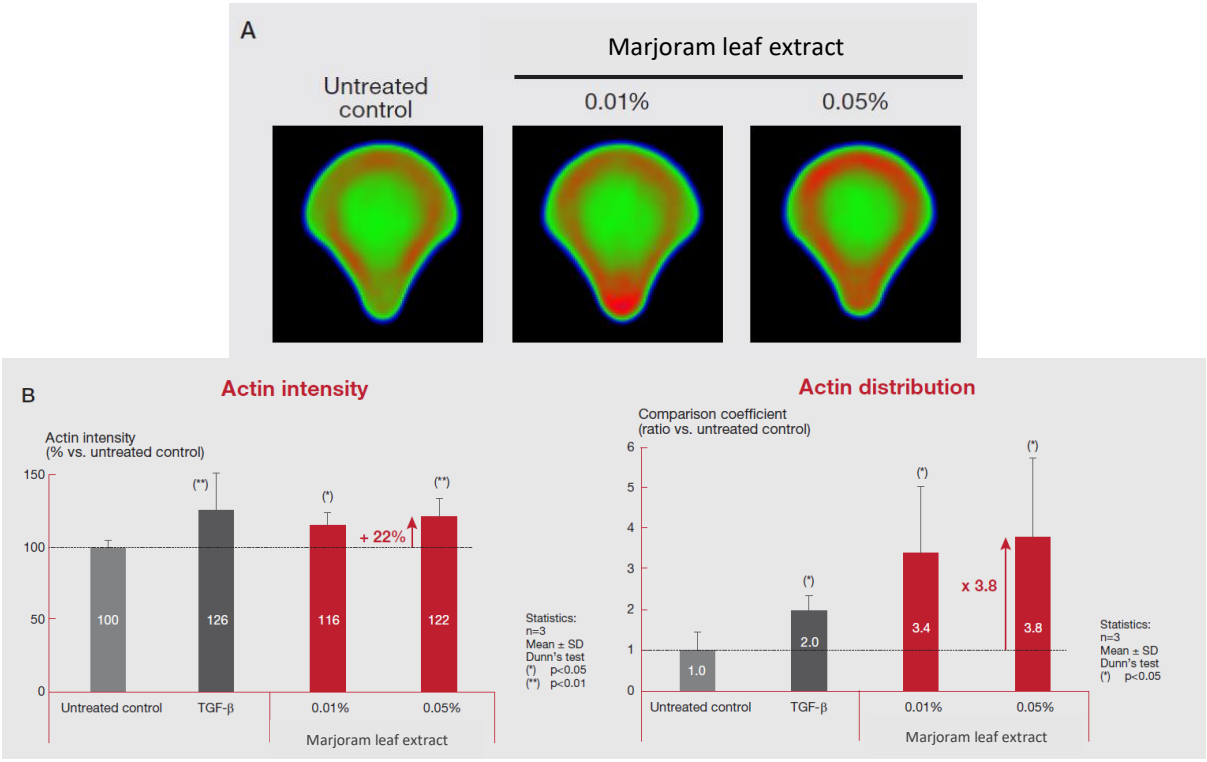
The purpose of this study was to first monitor Marjoram leaf extract's effects on actin stress fibres expression and distribution at the single cell level in fibroblasts knowing that actin is involved in both stretch and adhesion fibres. Secondly, the effects of Marjoram leaf extract were monitored on the contractility of a group of fibroblasts to restore dermis ECM integrity.

To this end, we used a unique and innovative platform to explore the effect of Marjoram leaf extract on actin network and contractility. Micropatterning technology allows the control of cell substrate geometry and stiffness. We used primary human dermal fibroblasts to measure the impact of Marjoram leaf extract on the F-Actin network (actin organisation) at the single cell level and at the level of a group of cells.

Results and Discussion

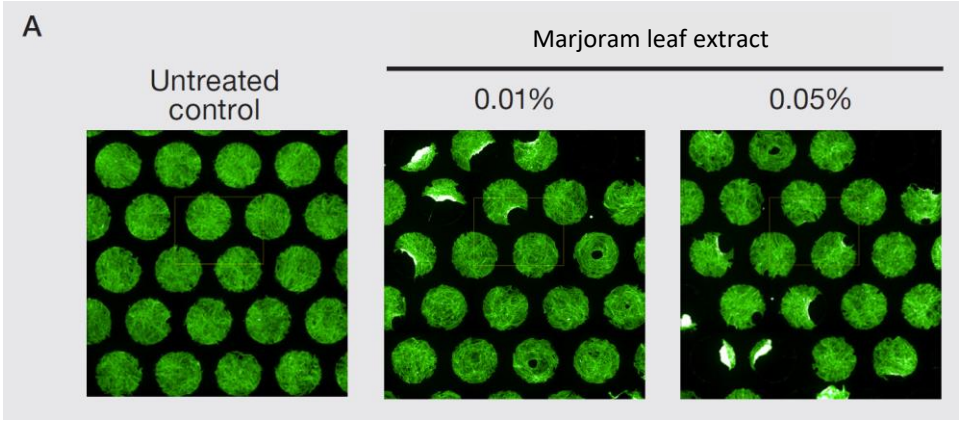
Reinforcement of intracellular structures at the single cell level.

In figure 5A, results showed that Marjoram leaf extract increased actin intensity and change actin distribution (more red colour than in untreated control). Quantification results in figure 5B showed that Marjoram leaf extract at 0.05% induced actin intensity by 22% and actin distribution by 3.8-fold. Marjoram leaf extract significantly reinforces stretch and adhesion-associated actin fibres between 0.01% and 0.05% Marjoram leaf extract (Figure 5).



Induction of Fibroblast contractility at a level of a group of cells.

Furthermore, results in figure 6 showed that Marjoram leaf extract contracts significantly dermal fibroblasts and increases actin network when tested from 0.01% and 0.05%. The more the fibroblasts contract, the more the fibroblasts come off the support and the less structures were counted which characterises the fibroblast contractility.



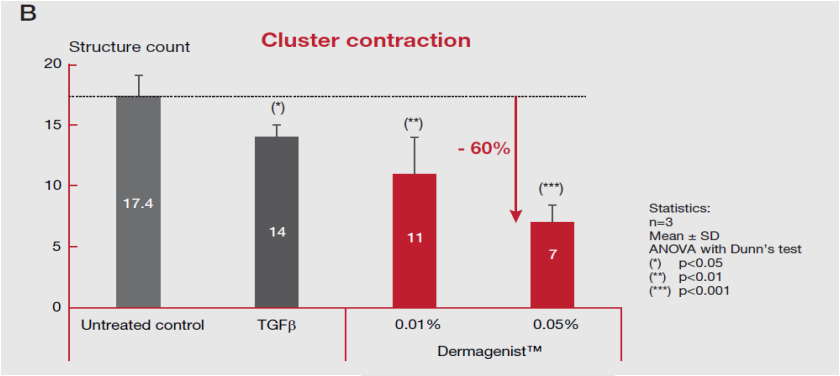


Figure 6 – Monitoring of the contractility of a group of cells. A) Representative images of fibroblasts islets in control untreated cells and in Marjoram leaf extract treated fibroblasts. B) Quantification of their entire structures.

Conclusion

By re-inducing actin synthesis and distribution in the fibroblasts, Marjoram leaf extract reinforces fibroblast intracellular structures and contractility. Marjoram leaf extract gives back to the fibroblasts, a new vitality and the ability to connect to the surrounding matrix.

Materials and Method

Cell culture

Human dermal fibroblasts from a female Caucasian donor of 57 years old are used at passage 4 for both assays. Experiments were performed in triplicate on each assay. Marjoram leaf extract was added at 0.01% and 0.05%. Both FibroScreen Actin and Contractility assays were automated. All pipetting handling (seeding, washing, compound addition) were performed using the TECAN Evoware platform, and all the washing steps during the immunostaining are done with a washer/dispenser instrument (BIOTEK).

FibroScreen Actin Assay

Quantification of single cell actin content of more than 150 single spread cells per well. The crossbow micropattern allows highlighting stress and adhesion actin fibres. A reference pattern of actin distribution and intensity is defined using adherent, untreated

control cells. Actin synthesis and distribution of treated cells was then compared to untreated cells. TGF-β was the positive control.

FibroScreen Contractility Assay

The FibroScreen Contractility assay is based on cell behaviour over the whole of the micropattern. The assay protocol includes cell seeding, Marjoram leaf extract addition, cell staining, and results analysis based on high content analysis and microscopic observations. Cell clusters with increased cell tension came off the support and the greater the number of loose clusters, the high the tightening effect and less structures were counted.

Statistics

Averages and standard deviations were calculated using 15 control wells, and 2 reference compound wells. An ANOVA analysis of variance in conjunction with a Dunn's test were used to assess significant differences between control and treated conditions: *p<0.05, **p<0.005, ***p<0.001.

Efficacy

Re-activate cells' machinery through epigenetic regulation

Objective

With ageing, cells are in a resting state due to a slackened cell metabolism. In the dermis, collagen and its ECM partners are downregulated leading to a loss of skin firmness and elasticity. Our aim is to re-induce the expression of genes by waking up the cells. Genes are partly switched off because of epigenetic regulation and particularly due to DNA methylation. Dismantle DNA methylation of the genes could be a way to wake up the cells and give the cells a new life to start over ECM synthesis. Building on our experience on skin ageing, we have developed the epigenetic model on expression and methylation of *LOXL1* during ageing. We have consistently worked on *LOXL1,* the key known elastin cross-linked enzyme. In order to re-induce the process of gene expression, we addressed DNA methyl of the specific gene.

In a previous study, we have shown the *LOXL1* gene expression is downregulated with ageing[10]. We hypothesised that this downregulation could be, in part, manged by epigenetic changes because its promotor contains a GC (guanine, cytosine)-rich proximal region (-323/-494 relative to start codon) prone to DNA methylation. As methylated DNA was shown to counteract promotor activity, we then compared the methylation status of *LOXL1* promotor from healthy elderly fibroblasts to young fibroblasts, and thus assigned DNA methylation as the mechanism responsible for *LOXL1* repression in elderly fibroblasts. Therefore, we have shown the *LOXL1* promotor was methylated by the DNMT3A methylase (data not shown). Experiments have been done in scientific collaboration and results have been published[11].

Results and Discussion

Marjoram leaf extract effects on *LOXL1* mRNA expression and *LOXL1* promotor methylation:

According to the prevision results based on the proof of concept, we have first studied the effects of Marjoram leaf extract on *LOXL1* mRNA expression in elderly fibroblasts. Secondly, we have studied the effects of Marjoram leaf extract on *LOXL1* promotor

methylation driven by DNMT3A. Marjoram leaf extract re-induced *LOXL1* mRNA expression by 66% (Figure 7A). Moreover, we showed that Marjoram leaf extract decreased promotor methylation (Figure 7B) leading to a recovery of the promoter activity (Figure 7C). Therefore, Marjoram leaf extract rejuvenates cell's epigenetic pattern and reactivates gene expressions.

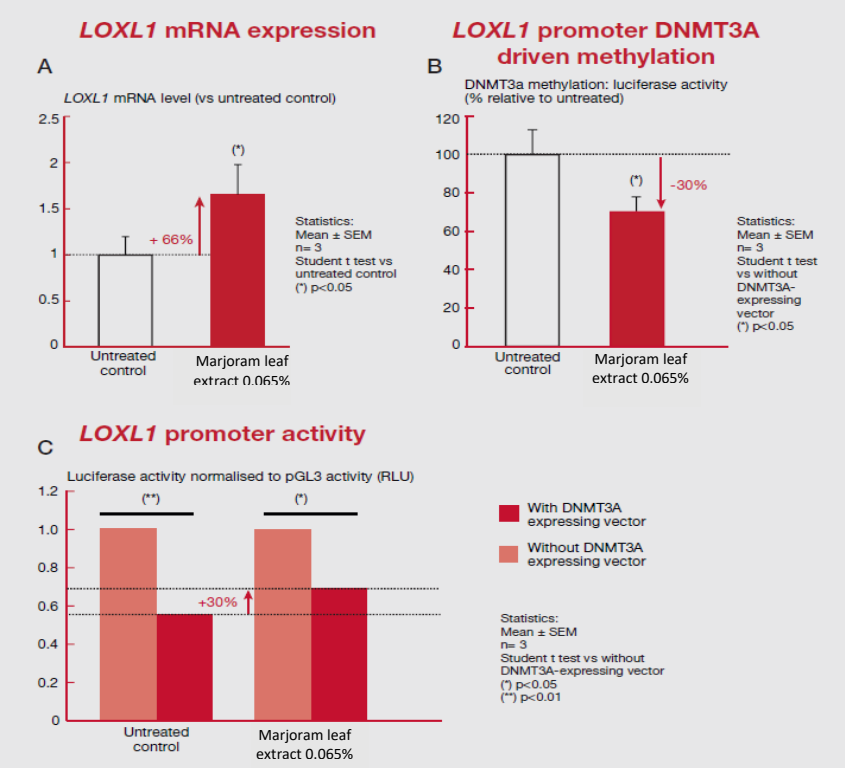


Figure 7 – Marjoram leaf extract effects of (A) *LOXL1* mRNA expression, (B) *LOXL1* promoter methylation, (C) *LOXL1* promoter activity through luciferase activity with or without DNMT3A.

We investigated for the first time the mechanism of *LOXL1* promotor regulation by epigenetic DNA methylation. We characterised the potential implication of DNA methylation in age-associated *LOXL1* silencing. As a consequence, we restored *LOXL1* mRNA expression with Marjoram leaf extract, by targeting such a mechanism.

Conclusion

By re-activating the cells' machinery, Marjoram leaf extract allows the fibroblasts to synthesise the extracellular matrix components they need.

Materials and Methods

Cell culture and real time RT-PCR

For the studies of *LOXL1* mRNA, experiments were carried out with fibroblasts from a 62-year-old donor. Cells were treated (or not) with Marjoram leaf extract at a dose equivalent to 0.065% for 2 days after confluence, and then the total RNA was extracted. Real-time RT-PCR assessments were performed with primers specific for *LOXL1* mRNA or for the housekeeping ACTB (coding for Actin B) and iTAQ Universal Sybr Green One Step Kit (Bio-Rad), according to manufacturer's instructions. Data were analysed using the ΔΔCt method. For the studies of the promotor activity, experiments

were carried out using Human Embryonic Kidney 295 (HEK 295) cell line in which new DNA can easily transfect within specific vectors.

Expression vector constructions
DNMT3A complementary DNA (cDNA) (NM-153759.2) was amplified by RT-PCR and the obtained fragment was cloned into pCep4 plasmid with Flag tag in C-terminus to produce the pcep-DNMT3A-Flag plasmid expressing an active DNMT3A enzyme. The *LOXL1* promoter sequence, obtained by PCR amplification from genomic DNA (gDNA), was cloned in front of the sequence coding for luciferase enzyme in plasmid pGL3 to obtain plasmid pLL32, expressing the luciferase enzyme under the transcriptional control of *LOXL1* promoter. We either transfected the cells with the plasmid containing DNMT3 (with DNMT3A) or with the plasmid which does not contain DNMT3A (control).

Luciferase activities
pLL32 construction was co-transfected with pCep4 (Mock) and pCep4-DNMT3A-Flag vectors in HEK 295. Transfected cells were treated or not with Origanum Majorana Leaf extract at a dose equivalent to 0.065% and lysed 24h post-transfection with a passive lysis buffer. Renilla luciferase (480nm) and Firefly luciferase (560nm) activities were assessed using Dual-Luciferase Report Assay System (Promega) and luciferase activity was analysed. Results were normalised to conditions of cell transfected with pRL-TK as transection efficiency reference and with pGL3 basic plasmid as background reference. The luciferase activity expresses *LOXL1* promoter activity.

Statistical analysis
The results are expressed as mean values ± SEM from triplicate assays or independent experiments as indicated in figure legends. The most appropriate statistical test for data comparison was selected from unpaired- or paired- Student's t tests, or one-way ANOVA test.

Efficacy
Re-build collagen network
Objective
The purpose of this study was to investigate the effect of Marjoram leaf extract, on collagen and its essential partner, BMP1, to get mature and functional collagen fibres in elderly fibroblasts. Marjoram leaf extract would then allow a recovery of a strong, thick functional collagen network in the dermis which otherwise is damaged with age. The studies were performed at mRNA and protein expression levels for collagen I and protein level for BMP1.

Results and Discussion
1. Marjoram leaf extract stimulates pro-collagen I expression at both mRNA and protein levels.
Results showed that Marjoram leaf extract at 0.015% induces collagen I mRNA expression by 89% (Figure 8A) and intracellular pro-collagen synthesis by 36% (Figure 8B). Results were significant compared to the untreated control.

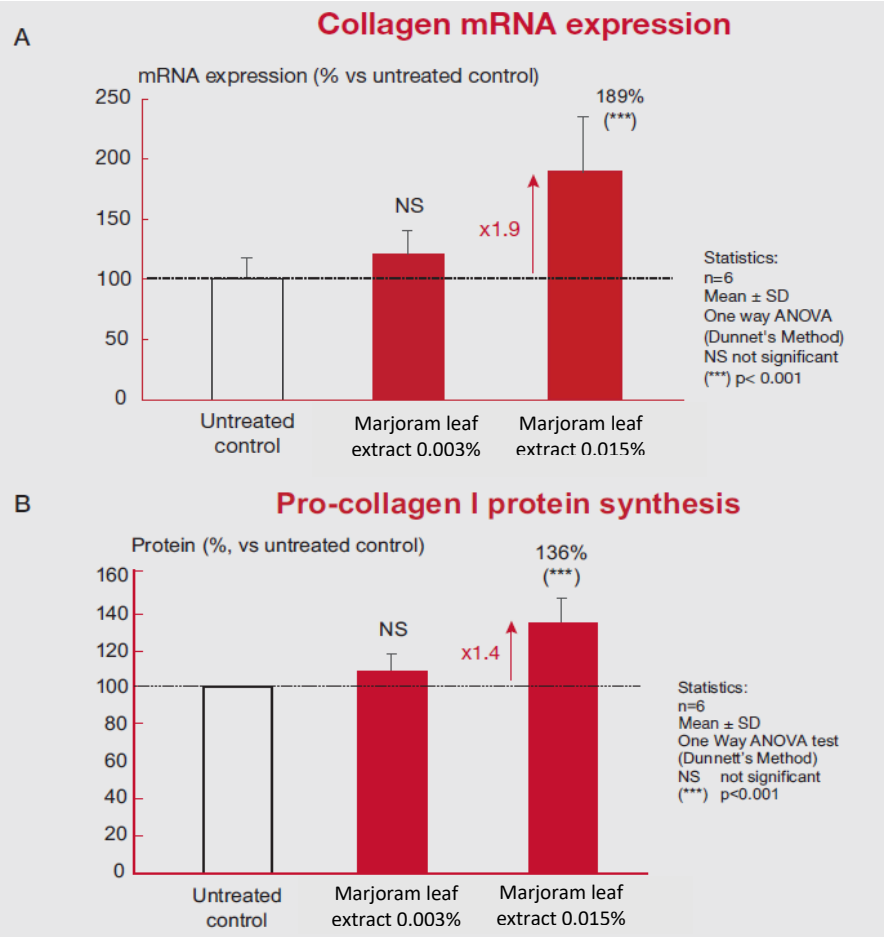


Figure 8 - Effect of Marjoram leaf extract on collagen I expression (A) Expression of collagen I mRNA in fibroblasts cultured either with or without Marjoram leaf extract. (B) Synthesis of intracellular pro-collagen I in fibroblasts cultured without (untreated control) or with *Origanum Majorana* leaf extract at a dose equivalent to 0.003% or 0.015%. Results expressed as % related to untreated cells, control (100%)

Materials and Methods

Collagen I mRNA quantification

Normal human dermal fibroblasts obtained from an abdominal biopsy from a 63-year-old were cultured in monolayers in 24-well plates and grown until confluence in a serum-free defined Fibroblast Medium for 96 hours at 37°C/5% CO_2 without (untreated control) or with *Origanum majorana* leaf extract at a dose equivalent to 0.003% and 0.015%.

Cells were washed with PBS then total RNA was extracted with an SV Total RNA Isolation System according to the manufacturer's instructions. Total RNA content and quality were evaluated by a measurement of optical density at 260nm and 280nm. Real-time RT-PCR was performed using iScript One-Step RT-PCR Kit with SYBR Green) on 50ng of total RNA as indicated by the manufacturer. Primers used for the PCR were the following:

LOXL1
Forward 5' - CTGGCCGCCATACTCGAAC - 3'
Reverse 5' - CAGAGGGAAGCCGCAAGA - 3'

Actin
Forward 5' - GTGGGGCGCCCCAGGCACCA - 3'
Reverse 5' - CTCCTTATTGTCACGCACGATTTC - 3'

All primers were located in separate exons. Amplification were performed on a Chromo4 with 40 cycles at 95°C for 5s, 60°C for 30s, 72°C for 30s, measuring the fluorescence at the end of each cycle. Melting curves were performed at the end of amplification to check the specificity of primers. The comparative Ct method (f¢Ct) was used for relative comparison. Real-time PCR experiments were calibrated with actin as housekeeping gene. As negative controls, samples without RNA were used in the same conditions.

Intracellular pro-collagen I synthesis
Normal human dermal fibroblasts obtained from an abdominal biopsy from a 63-year-old donor were cultured in monolayers I 96-well plates and grown until confluence in DMEM GlutaMAX medium added with 10% foetal calf serum, glucose, L-glutamine and antibiotics for 96 hours at 37°C/5% CO_2. At confluence, cells were incubated 48 hours at 37°C and 5% CO_2 in the presence of *Origanum majorana* leaf extract at a dose equivalent to 0.003% and 0.015%. After incubation with Marjoram leaf extract, the medium was discarded and dedicated lysis solution was added. Then the cell lysate obtained is recovered for Type I pro-collagen assay. Intracellular Type I pro-collagen secreted in the culture medium was assessed using a commercially available ELISA kit. A DNA assay was done on cell lysate to rationalise the results (result express intracellular procollagen I synthesis per cell).

Statistics
The results were expressed as percentage of Type I pro-collagen compared to untreated control standardised to 100%. Each condition was carried out in sixplicate (n=6). The statistical analysis was carried out using One-way ANOVA test (Dunnett's Method).

Results and Discussion
2. Marjoram leaf extract induces BMP1 synthesis:
To establish the action mechanism of Marjoram leaf extract, we observed that Marjoram leaf extract at 0.015% stimulated BMP1 synthesis by 3-fold in elderly fibroblasts. This increase of BMP1 associated to the simultaneous increase of collagen I production would allow an increase to the first step of collagen fibres assembly (Figure 9).

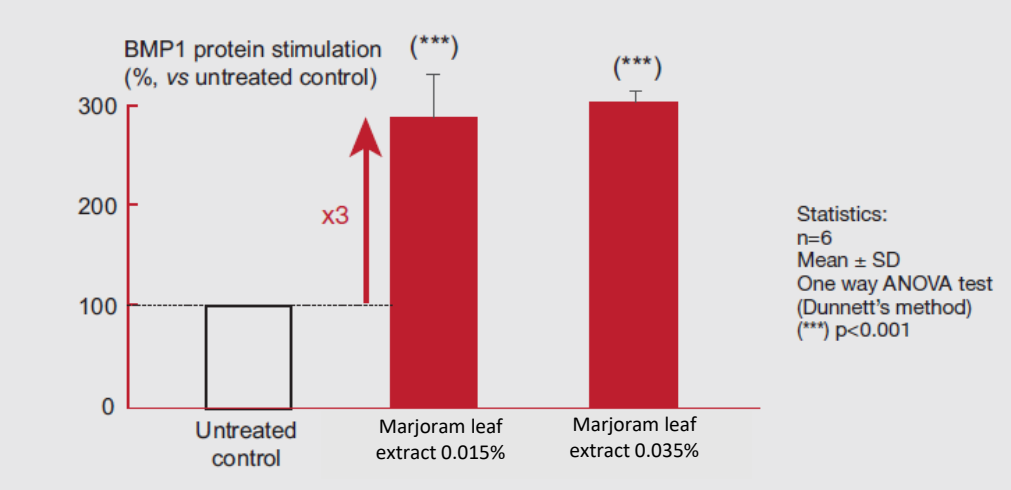


Figure 9 - Expression of BMP1 deposited protein in elderly fibroblasts cultured either without out with Marjoram leaf extract. Results expressed as % related to untreated cells, control (100%)

Materials and Methods

BMP1- synthesis

Normal human dermal fibroblasts obtained from an abdominal biopsy from a 63-year-old were cultured in monolayers in 96-well plates and grown until confluence in a serum-free defined Fibroblast Growth Medium for 96 hours at 37°C/5% CO_2. At confluence, cells were incubated 48 hours at 37°C and 5% CO_2 in the presence of *Origanum majorana* leaf extract at a dose equivalent to 0.003% and 0.015%.

After incubation with Marjoram leaf extract, the culture medium was collected for BMP-1 assay and dedicated lysis solution was added. BMP-1 secreted in the culture medium was assessed using a commercially available ELISA kit. Then the cell lysates obtained were recovered for DNA assay.

Results and statistics

The results were expressed as a percentage of BMP-1 compared to untreated control standardised to 100%. Each condition was carried out in sixplicate (n=6). The statistical analysis was carried out using One-way ANOVA test (Dunnett's Method).

Results and discussion

3. Effect of Marjoram leaf extract on collagen deposition:

As above results showed that Marjoram leaf extract stimulates collagen I at both mRNA and protein levels, and BMP-1 deposited protein, the following study analysed the effect of Marjoram leaf extract on deposited mature collagen in the ECM.

Marjoram leaf extract stimulates deposited collagen I synthesis compared to untreated control with a significant stimulation at 0.015% (138%). This result was as effective as Vitamin C positive control (Figure 10A). However, the collagen I stimulation obtained with Marjoram leaf extract at 0.03% is better than Vitamin C (158%).

Next, we looked at deposited collagen I fibres formed by fibroblasts in the presence of Marjoram leaf extract with immunostaining. The untreated control showed pro-collagen I staining in the intracellular compartment. After 3 days of treatment, we observed a stimulation of collagen I fibres into the extracellular matrix with Marjoram leaf extract at 0.015%. The stimulation effect occurred and fibres are localised while control fibroblasts do not show such deposition (Figure 10B).

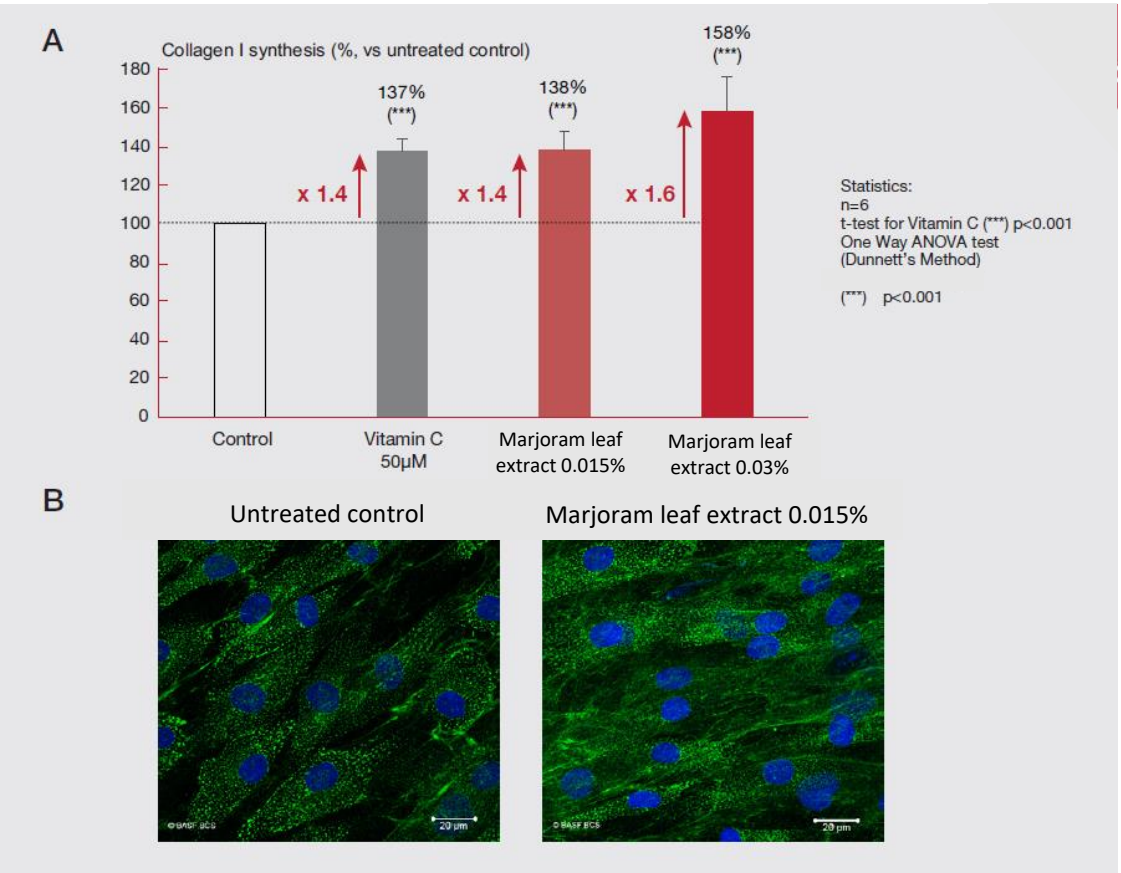


Figure 10 - (A) Synthesis of deposited protein collagen I in elderly fibroblasts cultured either without or with Marjoram leaf extract, Vitamin C (50µM), as a positive control. Results expressed as % related to untreated cells, control (100%). (B) Immunofluorescent labelling of Collagen I on fibroblasts in monolayers. Collagen I in green. Nuclei in blue. Scale bar = 20µm.

Materials and Methods

Collagen I deposition

Collagen I deposition was performed in the matrix of normal human fibroblasts using a Delfia method developed internally (Immunoassay based on a time-resolved fluorometric method, patented).

Fibroblasts of a 63-year-old donor were cultivated under confluence and then incubated for 72h 37°C, 5% CO_2 with *Origanum majorana* leaf extract at a dose equivalent to 0.03% and 0.015%. The medium was next discarded and dedicated lysis solution was added. This solution allows the disruption of cell membranes without solubilising the deposited matrix. Then the cell lysate obtained was recovered for DNA assay. In parallel, a PBS/BSA saturation solution was added onto the matrix. The primary anti-collagen I

antibody was added. After rinsing in PBS, the Europium-conjugated secondary antibody was added. Finally, a specific enhancement solution was added. Fluorescence intensity was read (λexc.340nm/ λem. 615nm) using an EnVisionλ multilabel plate reader.

Results and statistics

The results were expressed as a percentage of deposited collagen I synthesis compared to untreated control standardised to 100%. Each condition was carried out in sixplicate (n=6). The statistical analysis was carried out using One Way ANOVA test (Dunnett's Method) for Marjoram leaf extract and Student's t-test for Vitamin C.

Visualisation: Immunofluorescent labelling of Collagen I in fibroblasts in monolayers

Fibroblasts obtained from the abdominal surgery of a 63-year-old donor were seeded in 8-well culture chambers at the density of 23,000 cells per well. Cells were cultured until confluence in a serum-free, defined Fibroblast Growth Medium. Confluent fibroblasts were treated with 50µM vitamin C or *Origanum majorana* leaf extract at a dose equivalent to 0.015% with vitamin C 5µg/ml for collagen fibres induction for 3 days in basal medium FGM. At the end of the incubation period, cells were rinsed twice in PBS and fixed 10min in cold methanol before immunostaining. Primary antibody anti-Collagen I was incubated 1 hour at room temperature in moist chamber at 1/200 in 1% PBS-BSA. Then chambers were rinsed in PBS and incubated with secondary antibody couple to Alexa Fluor 488 goat anti-rabbit lgG, for 30 minutes at room temperature in a dark moist chamber. Chambers were rinsed in PBS and in water. Chambers were removed and slide mounted using ProLong Gold antifade reagent with DAPI staining in blue. Slides were observed using confocal laser scanning microscopy. Antibodies were detected at 488nm (green) and nuclei at 405nm (blue). Immunofluorescence images were taken with a confocal microscope.

Results and Discussion

4. Second harmonic generation (SHG): analysis of organised maturated collagen:

Once we have shown that Marjoram leaf extract induced deposited maturated collagen I, we checked whether the collagen produced was functional. In order to observe deeply the ECM components in the dermis, we used a microscopy technique combing (1) a multiphoton excitation, (2) the detection of fluorescence signals and (3) Second Harmonic Generation (SHG) signals. With this specific technique, we looked at all the collagens present in the skin on skin biopsies and on reconstructed dermis model.

Autofluorescence and SHG on normal human young and old skin biopsy.

In figure 11, we observed the autofluorescence (in red) of the skin in a normal human biopsy, and thanks to the SHG, we observed the collagen fibres in grey. We compared the organisation of the collage in young normal skin compared to an old skin. In old skin, we observed that the collagen network in broken, the density of the dermis is lost whereas in young skin, the collagen network is tight.

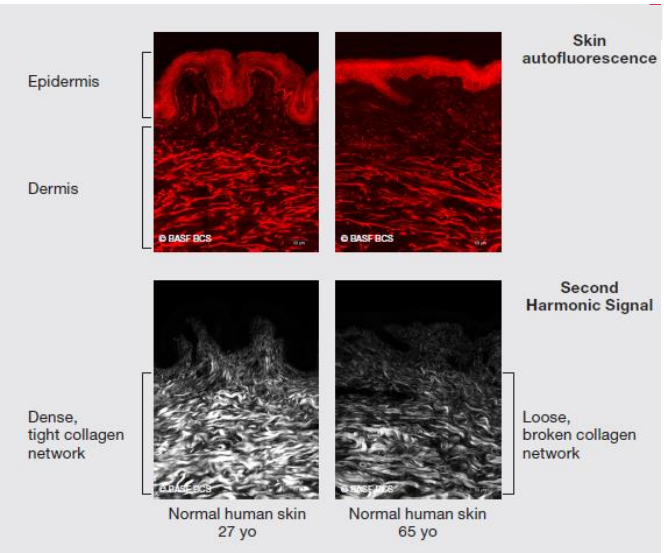


Figure 11- Visualisation of skin auto-fluorescence of the normal human skin (young versus old) in red in the upper part. Detection of functional organised collaged in a normal human skin in the lower part with Second Harmonic Signal (SHG).

Marjoram leaf extract effects on collagen visualised by microscopy (SHG).
Marjoram leaf extract was able to fill extracellular spaces with a fully organised three-dimensional collagen network, meaning that Marjoram leaf extract stimulated fibrillar collagen organisation shown in the dotted circle. In the untreated control and even in Vitamin C, the extracellular spaces are not filled with collagen. Then, Marjoram leaf extract is not only able to produce deposited collagen I on mature fibroblasts, but also able to produce mature and functional neo-synthesised collagen in reconstructed dermis as observed on the human skin biopsy (Figure 12).

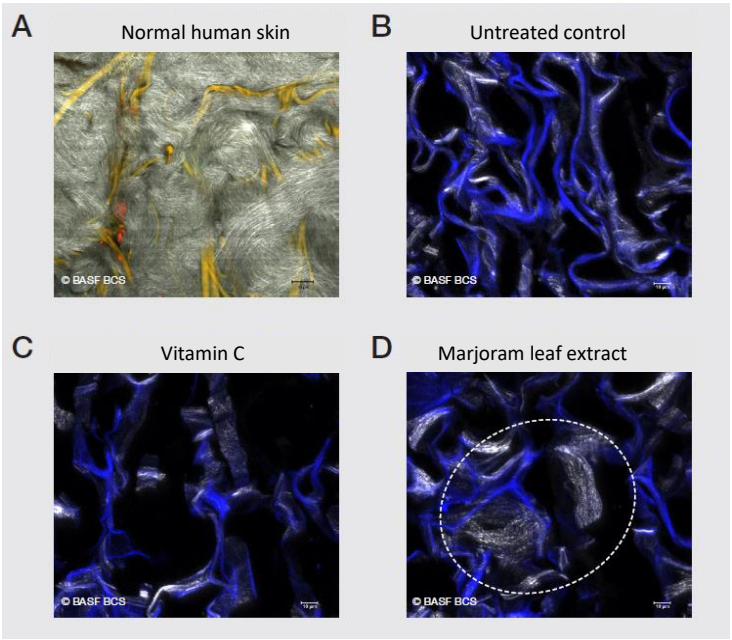


Figure 12 - On normal human biopsy (A) Two photon Excitation Fluorescence (TPEF) of elastic fibres in yellow and SHG of fibrillar collagen in grey. Reconstructed dermis model (B, C, D) collagen observation. TPEF of scaffold matrix in blue, and SHG of fibrillar neo-synthesised collagen in grey. Scale bar = 10µm.

Materials and Methods

Two Photon Excitation Fluorescence-Second Harmonic Generation: TPEF-SHG

Cell Culture

Normal human biopsies were analysed without being cultured. Vitamin C (50µM) or Marjoram leaf extract at 0.04% were added at day 4 after cells seeding for 24 days until the end of the culture.

Two-Photon excitation

We call the two-photon absorption when an atom or a molecular is able to absorb "simultaneously' two-photon (2P). To observe this phenomenon, the light intensity has to be very large and when the two photons arrive near the atom to excite, they have to be spaced by a sufficiently short time (<200fs). In these conditions, their actions add up. A pulsed laser system named COHERENT with a wavelength of 800nm (3300mW) is used to obtain an observation of the SHG signal at 400nm (BP Filter 390/400nm) as well as an auto-fluorescence over 405nm (LP405nm filter) was then used.

Second Harmonic Generation Signal.

The detection of the SHG signal, following a two-photon excitation and coming specifically from collagen, provides structural and non-invasive information, without exogenous staining of collagen having a triple helix structure (long helical domains, tropo-collagen).

Conclusion

Marjoram leaf extract restores the capacity of fibroblasts to produce mature and functional collagen in ageing skin by inducing the different steps of the collagen maturation process from the start of the expression of collagen mRNA in the cell's nucleus.

Efficacy

Strengthen cell-matrix interactions

Objective

Once fibroblasts are in a good shape, well activated (epigenetic regulation) fibroblasts are able to synthesise and stimulate ECM components in order to attach strongly to the matrix. Therefore, in the next experiments, we have checked the attached of the fibroblasts to a gel of collagen which mimicked physiological ECM. Indeed, a functionally intact cytoskeleton is crucial to build up contraction forces in a three-dimensional collagen lattice. These cell functions are essential for the homeostasis of the ECM. The crawling locomotion of the fibroblasts results from their exertion of contractile 'traction' forces on objects and materials to which they adhere.

Results and discussion

Cell-matrix interaction strengthening

We confirmed that that contracting effect of Marjoram leaf extract in fibroblasts by measuring a significant decrease in the micropattern area (figures 13A and 13B). Even if this contracting effect remains lower than the positive contracting control (TGFβ), the micropattern area is decreased by -4% compared to the untreated "control cells" condition. This contracting effect correlates with a strong increase in F-Actin intensity that was previously detected in Figure 4.

We can therefore conclude that Marjoram leaf extract stimulates the F-Actin network, increasing fibroblasts mechanical forces allowing attachment to the ECM.

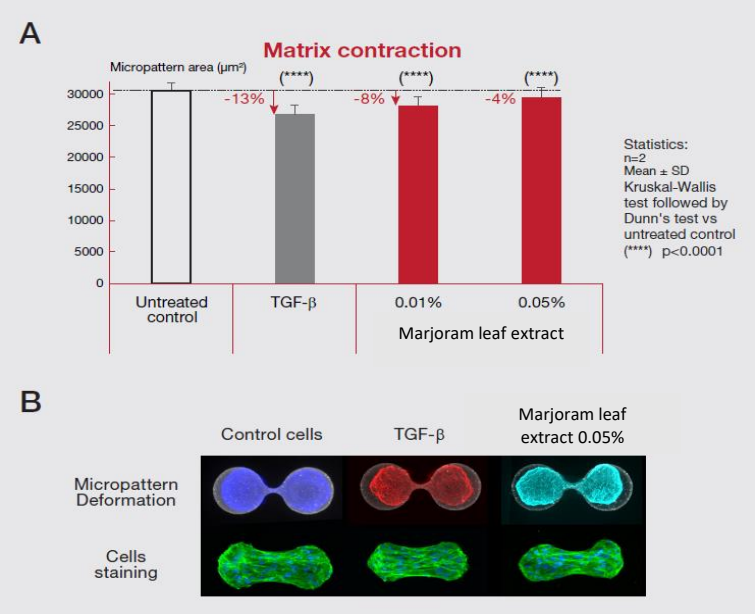


Figure 13 (A) Quantification of fibroblast-induced matrix contraction, (B) Micropattern images: in the upper line, in grey, micropattern area, in blue, area with fibroblasts, in red area with cells treated with TGF-β, and

Conclusion

Marjoram leaf extract strengthens cell-matrix interactions which are essential to both good health of cells and, to the synthesis of extracellular matrix components, including collagen.

Materials and Methods

Human dermal primary fibroblasts from a 57-year-old female donor are seeded at passage 6 on chips containing 1000 dumbbells micropatterns coated with fluorescent fibronectin. Cells are seeded on dumbbell shaped micropattern lying on top of 2kPa substrate coverslip. Upon adhesion fibroblasts contract and pull on the substrate intrinsically, leading to a reduction of the micropattern area. By contracting, Marjoram leaf extract added at 0.01% and 0.05% modify fibroblasts mechanical forces, thereby their ability to deform the substrate and to change the micropattern.

Statistics

Averages and standard deviations were calculated using all the micropatterns detected on the 2 chips duplications (820 < micropatterns, 1210). The statistical analysis was carried out using Kruskal-Wallis test followed by post hoc Dunn's test. n = 2, Mean ± SD, ****p<0.0001.

Conclusion *in vitro*

With Marjoram leaf extract, cells recover cytoskeleton organisation and metabolism activity to synthesise ECM components to a strong adhesion to the matrix.

Thanks to Marjoram leaf extract, fibroblasts recover a right cellular cytoskeleton which leads to a strong skin structure linked to a good dermis mechanical property. Since cells recover a dynamic shape, they are able to synthesis ECM protein partners to let them get better interactions with the ECM, thanks to an epigenetic control. Marjoram leaf extract is a cosmetic ingredient to fight against the skin ageing process and restore skin firmness using a global collagen booster approach including collagen synthesis, processing and maturation (Figure 14).

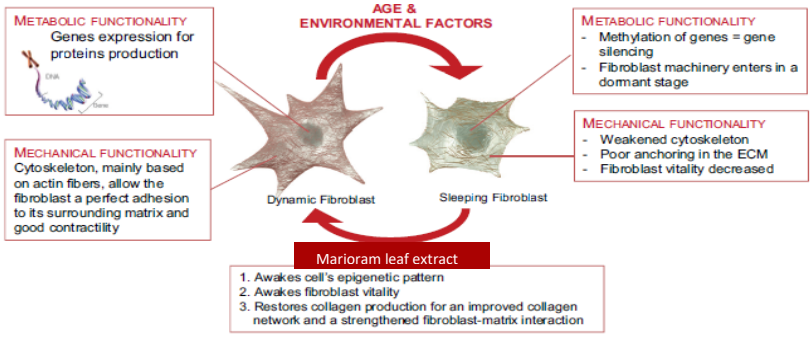


Figure 14 - Marjoram leaf extract awakens the cell's epigenetic pattern and fibroblast mechanical functionalities.

Efficacy

Improve skin density and firmness

Objective

2 clinical studies have been run. In placebo-controlled clinical studies, we first evaluated the ability of Marjoram leaf extract to increase skin density using an ultrasound system (Figure 15). Secondly, we evaluated with an expert evaluation the ability of Marjoram leaf extract to improve skin firmness (Figure 16).

Marjoram leaf extract 0.4%

Placebo cream

Figure 15 - Dermis density clinical study. Marjoram leaf extract vs. placebo. Schema of the protocol.

Marjoram leaf extract 0.4%

Placebo cream

Figure 16 – Firmness clinical study. Marjoram leaf extract vs. placebo. Schema of the protocol.

Results and discussion

1. Skin density increased:

With the emulsion containing Marjoram leaf extract at 0.4%, we observed a statically significant ($p<0.05$) increase of 18% of skin density versus baseline after 56 days application (Figure 17). For placebo, we observed only a trend of evolution ($p<0.1$), 1.5 times lower than for Marjoram leaf extract (not a significant difference).

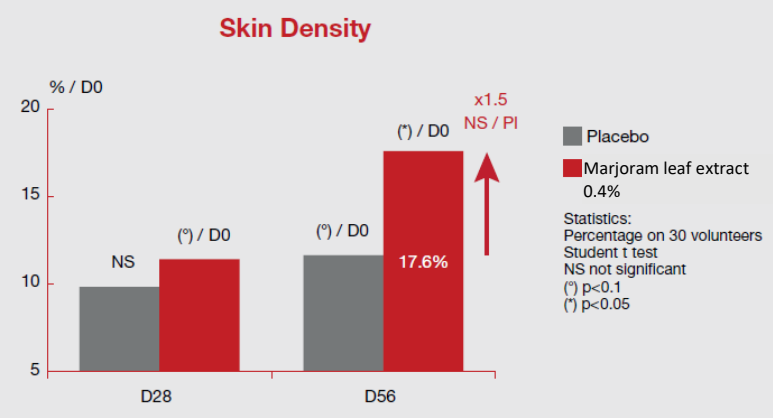


Figure 17 - Measure of skin density by echography on cheek bones.

Materials and method

Study design

The clinical study was carried out as a placebo-controlled double-blind randomised split-face study under dermatological control. The efficacy of the formulation containing Marjoram leaf extract PW BC10004 at 0.4% was compared to the baseline (before treatment, D0) and to the half-face treated with placebo. The study was conducted during a period of 56 days with check points at D0, D28 and D56.

Inclusion criteria

The study was done on 30 females, healthy volunteers, aged from 40 to 65 years old with phototype II, III or IV (Fitzpatrick) displaying low skin density (value measure by echography <25).

Application modality

The products were applied on cheekbones by the volunteers twice a day on each half face for 56 days, under the normal conditions of use.

Evaluation methods

The evaluation was done by echography. The DUBSkinScanner systems are high frequency and high resolution diagnostic ultrasound systems. It allows *in vivo* non-invasive skin analysis.

The DUBSkinScanner 75 with 50MHz probe allows very fine resolution of 31µm combined with maximum penetration of 4mm with Scan width: 12.8mm linear (33µm step width), with a level of amplification of the signal between 100 and 125% (the same for one subject), with viewing mode (B-scan). The software allows doing the

analysis of automatic skin density (corresponding to parameter: skin completely). An increase of the skin density value means an improvement of the skin density.

Statistics

The statistical analysis of the evolution of the parameters in function of time were done after the verification of the normality of distribution using Shapiro-Wilk test. Afterwards, the statistical analysis of the evolution of the studied parameters for each product was performed with the Student t test in case of the normality of the distribution had been confirmed. In case of the distribution had not followed the normal law, a non-parametric test Wilcoxon rank test) was used.

Results and discussion

2. Skin firmness improved

Marjoram leaf extract at 0.4% significantly increased skin firmness after 28, 56 and 84 days versus baseline, and after 84 days versus placebo (Figure 18). On average, the improvement of the firmness score increased with the duration of the treatment, reaching 15% over baseline ($p<0.001$) and +9% over placebo ($p<0.05$). Since 28 days of application, the first signs of the firming effect are perceived with on average an increase of 6.5% ($p<0.05$) with the Marjoram leaf extract formulation.

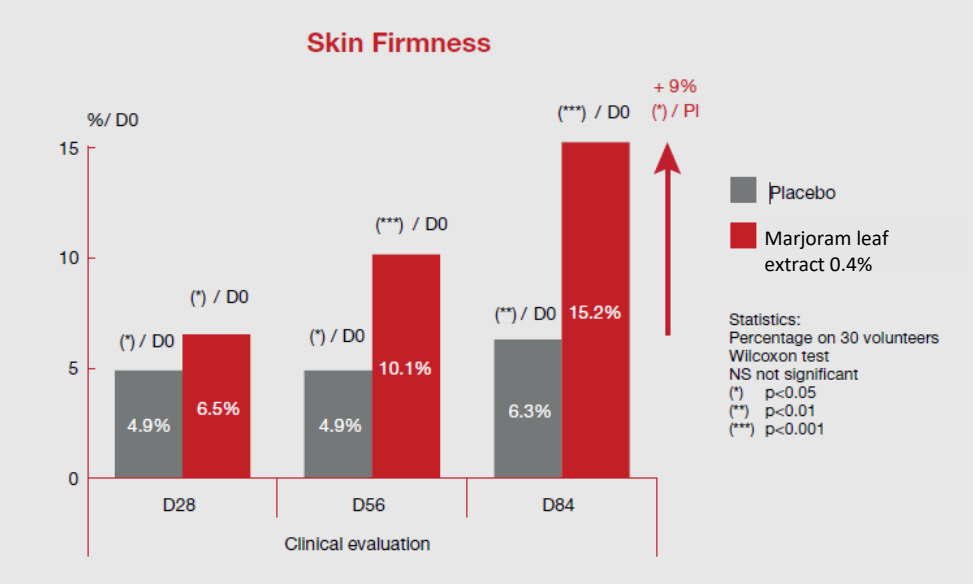


Figure 15 - Quantitative evaluation of skin firmness

Materials and methods

Study design

The clinical study was carried out as a placebo controlled double-blind randomised split-face study under dermatological control. The efficacy of the formulation containing Marjoram leaf extract at 0.4% was compared to the baseline (before treatment, D0), and to the half-face treated with placebo. The study was conducted during a period of 84 days with check points at D0, D28, D56, D84.

Inclusion criteria
The study was done on 30 females, healthy volunteers aged from 45 to 60-years old with phototype II, III or IV (Fitzpatrick).

Application modality
The products were applied by the volunteers twice a day on each half face for 84 days, under the normal conditions of use. Subjects were sitting in front of the assessor under controlled conditions (room temperature maintained at 21 '± 1°C and relative humidity at 45 ± 5%). The scoring was performed on cheeks. An increase of the score means an improvement of the skin firmness.

Evaluation methods
The evaluation was done by a trained assessor with scoring system in 10 points (0 to 9). The mode is tactile by pinching. The scale are as follows:

0 = not very firmed skin (flabby), easily stretching out of shape and not coming back quickly to its initial state.
9 = very firmed skin, skin having a clear resistance to stretch and easily coming back to its initial state.

Statistics
The statistical analysis of the parameters evolution in function of time were done after the verification of the normality of distribution using the Shapiro-Wilk test. Afterwards, the statistical analysis of the evolution of the studied parameters for each product was performed with the Student t-test in the case of the normality of the distribution had been confirmed. In case of the distribution had not followed the normal law, a non-parametric test (Wilcoxon rank test) was used.

Conclusion
Marjoram leaf extract improves the age-related signs with an increase in skin density and an improvement of skin firmness.

General conclusion
With ageing, the cellular machinery goes down with a decrease of fibroblasts in the dermis as a consequence, less collagen and ECM components are synthesised. Indeed, the cells are less active, then disorganised and finally lose their properties to adhere and contract together. In consequence, the general metabolism of the cells is altered.

In Vitro, Marjoram leaf extract, by targeting actin cytoskeleton, allows the cells to recover the right cellular traction forces on the ECM. As a consequence, fibroblasts recover a good adherence and contract together. By re-activating the cellular machinery, targeting DNA methylation, Marjoram leaf extract re-induces ECM components synthesis. With Marjoram leaf extract, collagen I is induced from mRNA level to matured, organised and functional collagen. Thanks to SHG, we showed that Marjoram leaf extract re-induces neo-synthesis of collagen and particularly in our *in vitro model.*

Tested *in vivo*, in a placebo-controlled study, Marjoram leaf extract, improved skin density and firmness.

Therefore, Marjoram leaf extract, is the right solution to wake up the sleeping beauty.

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