Communication

The Natural Centella asiatica Extract Acts as a Stretch Marks Eraser: A Biological Evaluation

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Article

The Natural *Centella asiatica* Extract Acts as a Stretch Marks Eraser: A Biological Evaluation

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Abstract: Stretch marks are far from being exclusively appearing on pregnant women and appear whenever the body experience a rapid growth. Into the dermis, collagen fibres are altered associated with a loss of orientation and the elastic network is disrupted leading to a fibrotic organisation. This results to epidermal tearing which produce skin lesions. *Centella asiatica* is a well-known medicinal plants rich in triterpenic actives molecules and traditionally used to treat wounds and help skin repair. The aim of this study was to evaluate *Centella asiatica* extract as a natural way to solve stretch marks concern and understand its mechanism of action. We have first evaluated the fibroblast's proliferation based on scratch assay model and their genes expression by RT-qPCR. At the *ex vivo* level, elastin fibres were quantified by immunofluorescence. Collagen fibre's orientation and their occupation of the dermis were analysed after sirius red staining and specific software analysis. We have proven that *Centella asiatica* has stimulated fibroblasts proliferation, reduced extracellular matrix degradation and fibrosis. On stretch marked skin explant, *Centella asiatica* increased the occupation of collagen fibres and elastin production. Based on the mechanisms behind the formation of stretch marks, *Centella asiatica* restores dermis network by optimising fibres organisation for a visible skin remodelling effect.

Keywords: *Centella asiatica*; stretch marks; skin repair; *striae*

1. Introduction

Stretch marks, also called *striae* are a form of tissue damage on the skin due to an excessive stretching of the dermis [1,2]. Stretch marks are commonly associated with pregnancy and affects between 60% and 90% of women during their pregnancy [3]. In reality, they occur as the result of tearing of the dermis during periods of rapid growth of the body, or some body parts. Therefore, around 27% of adolescent are concerned by stretch marks [3]. As they are caused by the sudden expansion of the skin, especially in areas where fat is most likely to be stored in our body, thereby obesity may lead to the apparition of stretch marks. Stretch marks are thus usually found on the breasts, thighs, belly, especially near the navel, upper arms, underarms and on the lower back, both in women and men.

Stretch marks affects the dermis by preventing the fibroblasts from organizing collagen fibers to keep up with the skin's stretching. The collagen bundles are altered, they loss their orientation and organized into a fibrotic structure while the elastic network is also disrupted [4]. As a result, the skin appears less firm, less deformable and less plastic. This normally leads to epidermal tearing which can produce a lesion in the form of a visible stretch mark. Stretch marks could be classified into 6 different types depending on their appearance and epidemiology; *striae atrophicans* (thinned skin), *striae gravidarum* (following pregnancy), *striae distensae* (stretched skin), *striae rubrae* (red), *striae albae* (white), *striae nigra* (black), and *striae caerulea* (dark blue) [5]. Over time, stretch marks tend to atrophy and lose pigmentation, so depending on how recent they are on the skin. Thus, a hyper-pigmented stretch marks related to an acute stage characterized by the initial erythematous and a chronic stage is characterized by a hypo-pigmented and atrophic lesion [6].

Current treatment involved invasive method such as laser therapy, light therapy, collagen injection, laser lipolysis, radiofrequency techniques and microdermabrasion [1,6,7]. Topical solution may be used and among these, formulation with *Centella asiatica* has been described to improved stretch marks but its mechanism of action is poorly described [8–11]. *Centella asiatica* (CAST) is one of

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the most known traditional medicinal plants. It has long been used to promote skin repair and wound healing, as well as it is a medicinal remedy present in the Ayurvedic system and the Traditional Chinese Medicine [12]. It grows aside of cultivated fields in Madagascar and other Asian and African countries. It expresses, in its tiny and round leaves, as secondary metabolites, some biologically active triterpenic molecules (Asiaticoside, Asiatic Acid and Madecassic Acid) [13]. *Centella asiatica* is a promising candidate to solve stretch mark concern as it enhanced cells production and matrix component and it improves tensile strength [13]. Clinical study has shown that application of formulation with *Centella asiatica* significantly improved stretch marks appearance [9,14]. We have also previously conducted a clinical study on 54 women divided into two groups (the control group applying a placebo formula without *Centella asiatica* and a group using a formulation with *Centella asiatica* CAST) [15]. The volunteers used the product three times per day for one month. After four weeks using the CAST formulation, skin thickness in the edge and in the center of the stretch marks was significantly increased in comparison to the placebo, such as skin vascularization and elasticity for a visual reduction of stretch marks appearance [15]. Following this preliminary work, we wanted to go further on the mechanism of action of *Centella asiatica* and the aim of this study was to unveil the pathways impacted by *Centella asiatica* at the *in vitro* and *ex vivo* level.

2. Materials and Methods

The aim of this study was to evaluate the active ingredient obtained from *Centella asiatica* (Supplied by Givaudan Active Beauty) as a new way to solve stretch marks concern. This natural extract has a very fixed composition and is composed by Asiaticoside (about 10-16%), Asiatic Acid (about 6-9%) and Madecassic Acid (about 10-14%).

2.1. Wound healing on fibroblast

Fibroblasts (NIH-3T3) were maintained in supplemented DMEM medium containing 10% FBS and 1% antibiotics (DMEM complete medium) at 37°C 5% CO2. NIH-3T3 (105 cells/well) were seeded in 96-well Essen ImageLock plates (Essen BioScience) and grown to 80% confluence in a CO_2 humidified incubator. After 24 h, the scratch was made using the 96-pin WoundMaker (Essen BioScience). Fibroblasts were treated with *Centella asiatica* at 1, 5, 10, 25 or 50µg/mL. FBS 10% was used as positive control. Wound images were taken every hour for 36 hours, and the data analyzed by the integrated metric Relative Wound Density part of the live content cell imaging system IncuCyte HD (Essen BioScience). The experiments were done in triplicate wells.

2.2. Transcriptomic analysis on fibroblast

Normal human dermal fibroblasts (NHDFs) isolated from non-stretch marked or from stretch marked area from the same donor were seeded at 300 000 cells per well in 6-wells plate. After 48h of culture, NHDFs were rinsed two times with PBS and allowed to rest in FCS-free medium overnight before stimulation. Cells were stimulated with *Centella asiatica extract* at 10µg/mL versus untreated condition. After 24h of stimulation, total RNA was extracted by Extract-all method. RNA quality was controlled and a reverse transcription was performed to obtain cDNA. RT-qPCR was made on specific plates designed to study transcriptomic expression of different genes involved in stretch marks with 10 ng of cDNA per well. The results of gene expression obtained with fibroblasts were normalized according to POP4 (Ribonuclease/MRP subunit) and B2M (Beta-2-Microglobulin) housekeeping genes. The data are expressed in Fold change relative to normal fibroblasts or to untreated stretch marked fibroblasts.

2.3. Collagen network analysis on skin explant

Skin explant were obtained according to ethic and regulatory rules and under people agreement. Skin explants with stretch marks from a 60 years old volunteer donor have been treated topically with *Centella asiatica* at 0.5% for 5 days. Each day, treatment and medium (Genoskin) were renewed and explants were incubated at 37°C 5% CO2.

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INCI placebo: AQUA/WATER, CETYL ALCOHOL, GLYCERYL STEARATE, PEG-75 STEARATE, CETETH-20, STEARETH-20, ISODECYL NEOPENTANOATE, PHENOXYETHANOL.

After 5 days, skin biopsies were fixed in formalin (Sigma) for 48 hours and then dehydrated overnight (Leica). After dehydration, skin explants were included in paraffin and skin sections of 4µm were obtained with a microtome (Leica). Paraffin was removed thanks to xylene baths followed by ethanol dehydration. Skin slices were stained with a ready to use sirius red coloration (Labo Modern) before mounting on a thin glass slide. Dermis structure was observed using bright field and collagen fibers orientation has been analysed in polarized light. Under polarized light, collagen I fibres were detected using the red channel of slide scanner (Olympus). Quantity of collagen I was quantified using ImageJ software on the whole dermis. Besides, the images were oriented epidermal part up at 90° of the x-axis. The images were then processed to remove a 200µm thickness from the outer-most stratum corneum to eliminate the papillary dermis for the orientation analysis of collagen fibers. The conditioned images were processed by a segmentation algorithm allowing to focus the analysis only on the collagen fibers. Based on a parallel plan to the epidermis, collagen I fibers have been quantified at 10° intervals in order to evaluate collagen I occupation in the dermis.

2.4. Elastin immunofluorescence on skin explant

The skin explants were cultivated and treated as previously described. Paraffin was removed thanks to xylene baths followed by ethanol dehydration. Antigenic sites were revealed with a citrate buffer. Then, skin sections were BSA-saturated for 30 minutes followed by immunostaining to detect specifically Elastin (1/75, Santa-Cruz). After overnight incubation of the primary antibody at 4°C, skin sections were washed and incubated for 1 hour with secondary antibody (Anti-mouse, 1/500, Abcam). The staining was observed using fluorescent microscopy (Lordil Zeiss, Axio observer). Relative fluorescence was quantified for all images using ImageJ software.

2.5. Statistical analysis

For all studies, a Shapiro Wilk test was used to verify whether the raw data followed the Gaussian Law. In case of Normally-distributed data, the mean values were compared using an unpaired t student test. In case of non-Normally-distributed data, a Mann-Whitney U test was used. Whatever the used statistical test we considered significant results as following # $p<0.1$, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3. Results

3.1. Centella asiatica effect on fibroblast's migration

NIH-3T3 monolayer cell cultures were wounded and then treated with non-cytotoxic concentrations (1, 5, 10, 25, and 50 µg/ml) of CAST. CAST clearly induced wound healing in NIH-3T3 fibroblasts, even at the lower concentration tested. After 36 hours, CAST induced around 85% of wound closure regardless the testing concentration between 1 and 25µg/mL. However, at the higher concentration tested (50 µg/ml) CAST significantly inhibited the basal wound healing in NIH-3T3 cells (Figure 1). On the illustrative pictures, the proliferation of untreated cells was lower after 24hours in comparison to the cells treated with the FBS at 10% (which validated the experiment) and CAST at 10µg/mL.

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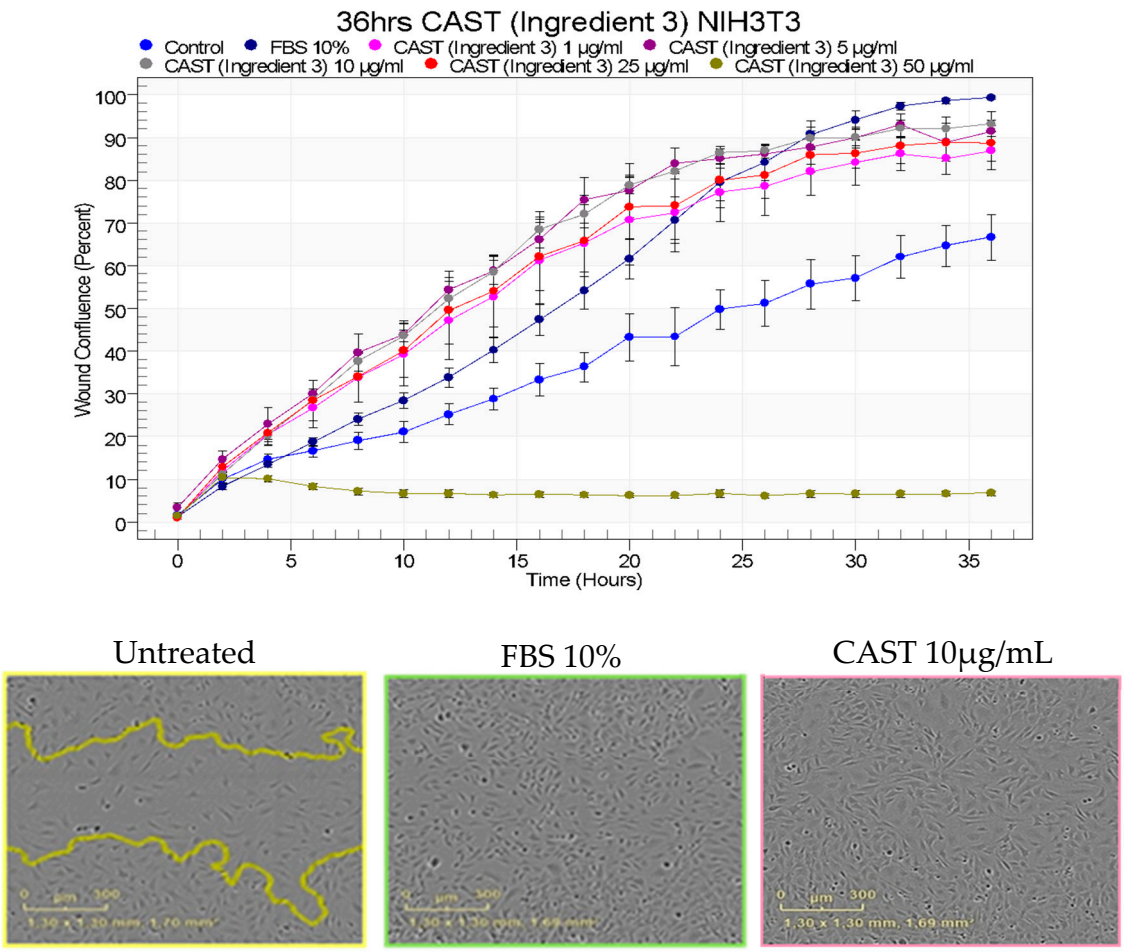

Figure 1. Effect of CAST on NIH-3T3 in wound healing. Wound closure kinetic after 36 hours of treatment with FBS 10% (positive control) and CAST at 1, 5, 10 and 25µg/mL starting just after the injury (top). Representative pictures of heal after 24 h following the treatment with FCS 10% (positive control) and CAST at 10µg/mL (bottom).

3.2. Anti-fibrotic effect of Centella asiatica

Stretch marked fibroblasts have been treated with CAST at 10µg/mL for 24 hours. An RT-qPCR analysis was performed with focus on matrix preservation and fibrosis markers. In comparison to normal fibroblasts, stretch marked fibroblasts significantly increased fibrosis markers by 63% and 66% regarding the expression of CTGF and PTK2 respectively. About matrix remodelling, MMP1 and MMP7 were significantly increased by 873% and 1230% respectively in comparison to normal fibroblasts. After CAST treatment, fibrosis and matrix degradation markers were significantly decreased by 128%, 58%, 149% and 50% for CTGF, PTK2, MMP1 and MMP7 respectively in comparison to the stretch marked fibroblasts (Table 1).

Table 1. Gene expression (fold change) on NHDFs from non-stretch marked donor or from stretch marked donor. Fibroblasts with stretch marks were treated with CAST at 10µg/mL. Unpaired Multiple T test with # p<0.1, * p<0.05, ** p<0.01 and *** p<0.001.

	Genes	Fold change Stretch marks *versus* un-stretch marked	Fold change CAST *versus* stretch marks
Fibrosis	CTGF (CCN2)	x1,6*	X0.44**

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	PTK2 (FAK)	x1.7#	X0.63**
Extra Cellular Matrix	MMP1	x9.7*	x0.40**
	MMP7	x13,3***	x0.67*

On stretch marked skin explant, the fibrosis is visible in bright field after sirius red staining and the accumulation of collagen I deposit is revealed by an intense red staining and an anarchic orientation of collagen bundles. The treatment with CAST reduced the fibrosis and collagen bundles are well organized. Moreover, the special sirius red staining highlights the natural birefringence of collagen fibers when exposed to polarized light. Collagen type I would show a yellow-red color and the quantification has shown a significant increase of collagen for stretch marked area in comparison to un stretch marked region by 10%. After treatment with CAST at 0.5%, the quantity of collagen I was significantly reduced by 10% in comparison to untreated stretch marks. The placebo has shown a strong impact on collagen and reduced by 18% in comparison to stretch marks. CAST was able to increase the amount of collagen I in comparison to placebo, reaching a normal collagen quantity (Figure 2).

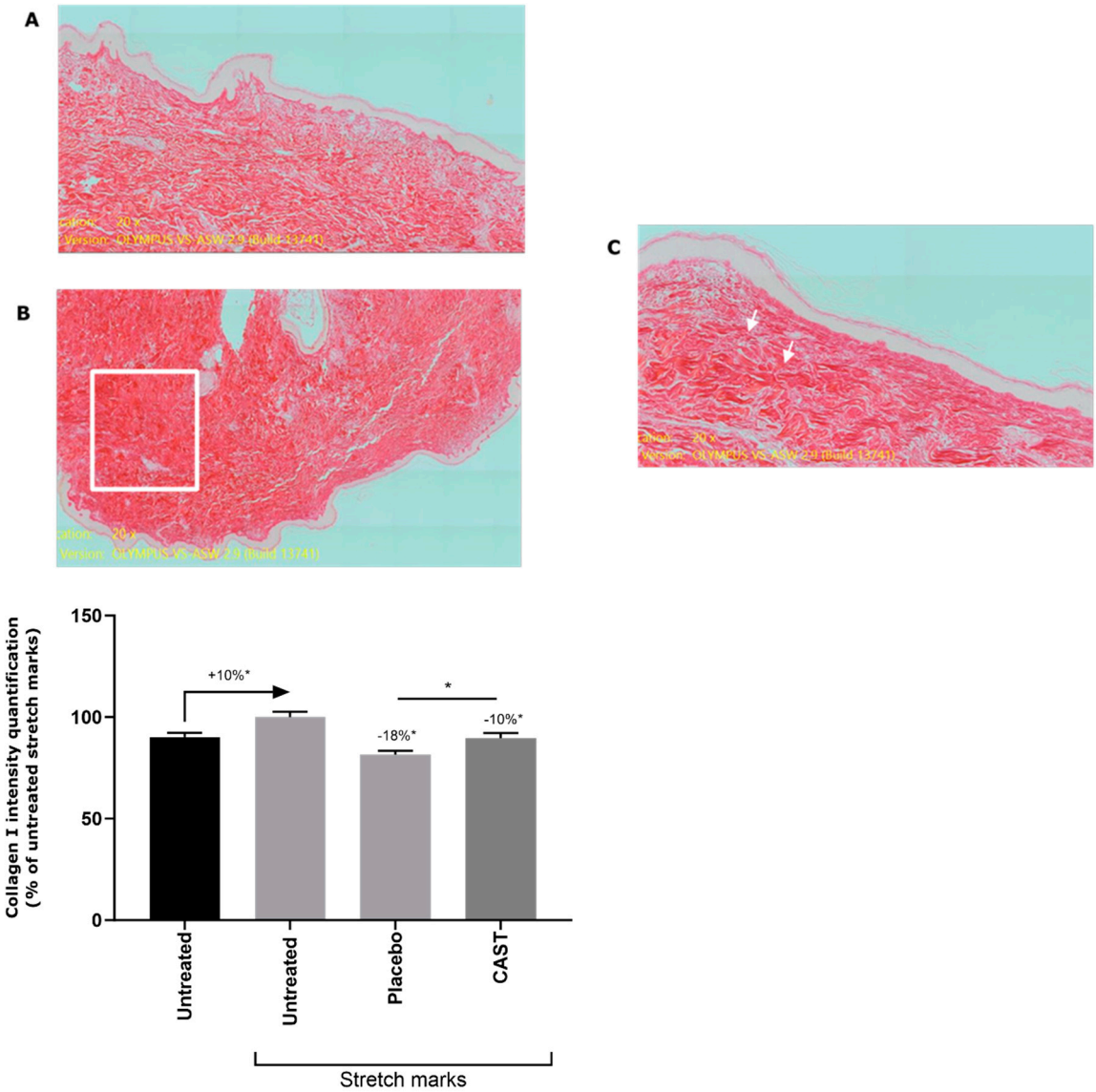

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Figure 2. Effect of CAST on skin fibrosis. Dermis network visualization in bright field (x20) after sirus red staining (top). (**a**) control, (**b**) untreated stretch marks skin explant and the square highlight a fibrotic organization (**c**) CAST 0.5% treatment, the arrows illustrates normal collagen bundles. Quantification of collagen I quantity in the whole dermis after sirius red staining observed under polarized light (bottom).

This special sirius red staining highlights the configuration of the collagen and the heterogeneity of the direction of the fibers in the tissues too. Image analysis after polarized light acquisition allows the quantification of the number of collagen bundles in each direction starting from a parallel plan to the epidermis. Stretch marks significantly decreased the occupation of the collagen fiber in the dermis by 33% in comparison to the normal skin. CAST at 0.5% significantly increased their occupation by 49% in comparison to the stretch marks explants (Figure 3). Besides, stretch marks impacted the preferential direction of the fibers especially between 20° and 60° relative to the epidermis. After treatment with CAST 0.5%, the active ingredient has restored this preferential direction of the collagen bundles (Figure 4).

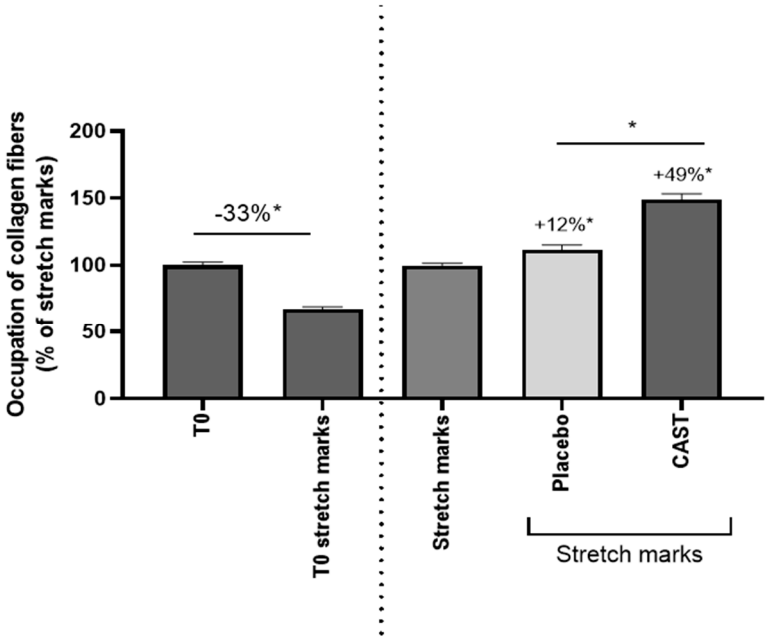

Figure 3. Collagen I fibers occupation in the dermis after sirius red staining and analysis in polarized light. Mann Whitney test with * $p<0.05$.

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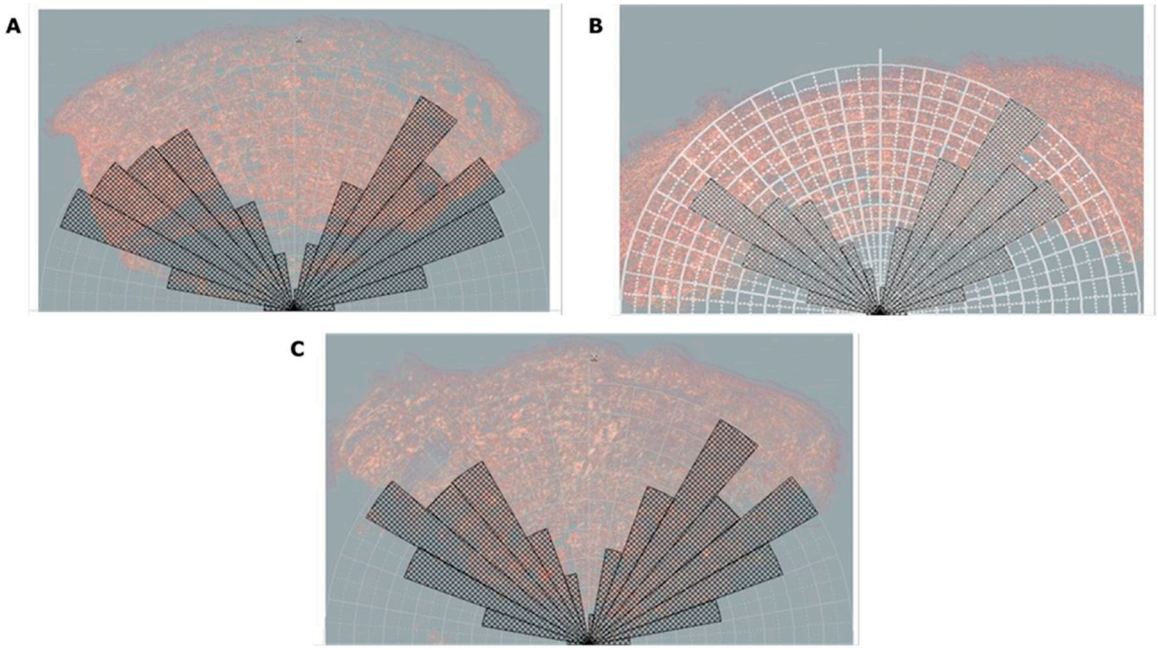
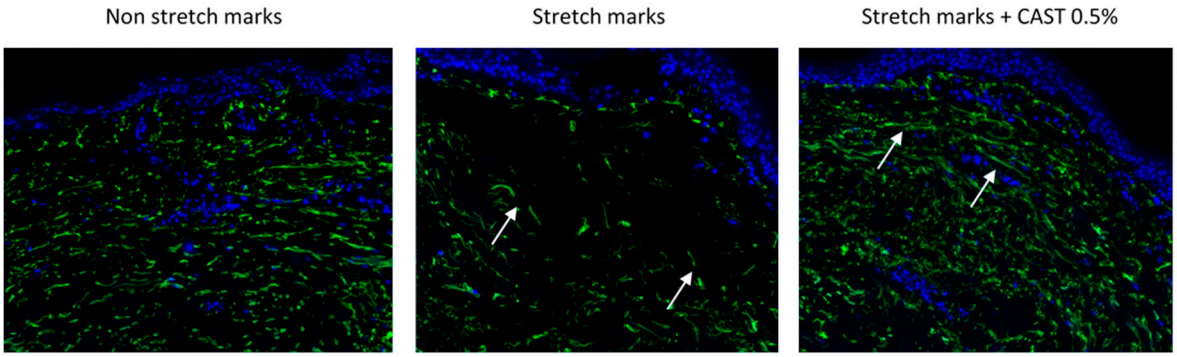


Figure 4. Collagen I orientation in the dermis. The fibers were quantified each 10° based on a parallel plan to the epidermis. (**a**) un-stretch marked skin, (**b**) untreated stretch marked skin and (**c**) stretch marked skin explant treated with CAST at 0.5%.

3.2. Centella asiatica improves skin elasticity

Elastin network was stained by immunofluorescence on human skin sections. The elastic fibres significantly increased by 11% in normal skin in comparison to stretch marks. After treatment with CAST 0.5%, elastin significantly increased by 37% in comparison to the untreated stretch marked skin (Figure 5).



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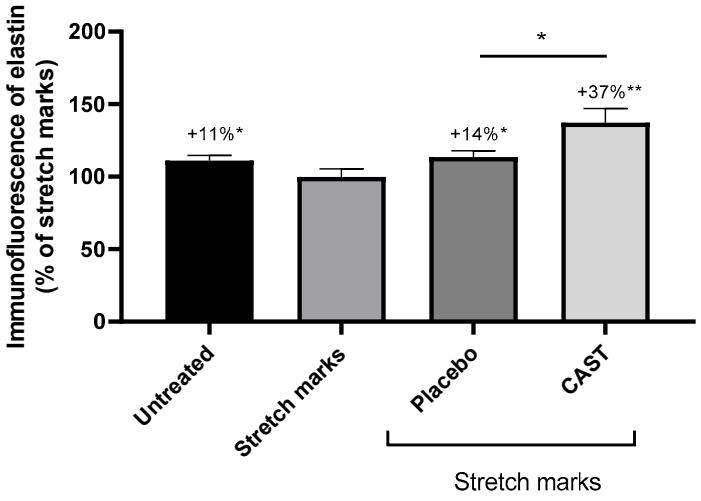

Figure 2. Elastin immunofluorescence visualization and quantification. The arrows indicate, in the case of stretch marks, a damaged and short elastic fiber whereas the treatment with *Centella asiatica* increased elastin content and fibrils maturation. Mann Whitney test with * $p<0.05$, ** $p<0.01$.

4. Discussion

Stretch marks concern are mainly characterized by a fibrotic skin. We have shown that fibroblast isolated from stretch marked tissue expressed fibrosis marker (CTGF and PTK2) leading to an intense sirius red staining on stretch marked skin sections. *Centella asiatica* has been described to promote *in vitro* fibroblast migration, matrix component and collagen synthesis [13,16]. Interestingly, during our study we have confirmed that *Centella asiatica* enhanced fibroblast migration but on stretch marked skin explants, the quantity of collagen I wasn't impacted by the treatment. We hypothesized that the effect of *Centella asiatica* at the *in vitro* level may be different than the effect on *ex vivo* skin explants depending on the physio-pathological environment and complex markers expression. For example, previous study using animal models has shown that *Centella asiatica* fasten skin healing by formation of a thick epidermal layer but with a moderate formation of granulation tissues and collagen which corroborate our observation on human [12,17]. In a context of pulmonary fibrosis, *Centella asiatica* was able to decreased fibrosis by decreasing collagen I content as well [18]. However, among the quantity of collagen, the orientation and the maturation of the collagen beams are essential. Using three-dimensional video, a study has demonstrated that collagen bundles are disrupted in stretch marks with marked separation of bundles and disorganization of collagen fibrils leading to this fibrotic organization [19]. We have shown using specific software which quantify collagen fibers in all the direction that the occupation of the dermis by collagen bundles was significantly improved following treatment with *Centella asiatica*. Finally regarding the collagen, *Centella asiatica* was able to promote the maturation and to restore the ori-entation of the bundles without enhancing the quantity of collagen. The elastic fibre network is also disrupted in stretch marks associated to microfragmentation, and immature elastic fiber newly formed are found in those gap [20–22]. By increasing elastin synthesis, *Centella asitica* is able to promote elastic fiber network, and staining analysis has shown the restauration of longs, thick and matures elastic fibres. Indeed, *Centella asiatica* was able to reduced fibrotic genes expression and also matrix degradation by MMPs to prevent this anarchic dermis organization. Likewise, our study has revealed that by promoting dermis organization among quantity of components, *Centella asiatica* works on stretch marks physio-pathological pathways.

5. Conclusions

Centella asiatica acts as a natural remodeling partner useful to solve stretch marks concern. Our study has unveiled the mechanism of action behind the efficacy of *Centella asiatica*. This traditional

key plant significantly reduced fibrotic markers and extra cellular matrix degradation. By decreasing collagen I degradation, the occupation of the beams and their preferential direction was restored for a pumpling effect in the center of stretchmark. *Centella asiatica* has also stimulated micro-circulation and tropo-elastin synthesis to fully replenish dermis net-work which lead to a clinical improvement, previously published, of skin elasticity and a visible erasure of stretchmarks.

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