A new anti-cellulite kit:
adipocyte differentiation and lipolytic activity

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Abstract

Two main mechanisms are involved in the development of the fat mass of adipose tissue:

- A well-known mechanism is an imbalance between the processes of lipogenesis and lipolysis in differentiated fat cells.
- Another mechanism is the adipogenesis, which leads to the transformation of preadipocytes in differentiated adipocytes. The newly formed mature adipocyte has acquired the capacity to store triglyderides in its lipid vacuole.

We have shown, using RT-PCR method, that the slenderizing active ingredients inhibits the differentiation of preadipocytes into adipocytes by reducing the expression of mRNA coding for PPAR-γ which is a key transcription factor without which the differentiation of preadipocytes cannot occur. Moreover, the slenderizing active ingredient stimulates the lipolytic activity of mature adipocyte. This effect could be compared to the caffeine.

Finally, tested directly *in vivo*, the slenderizing active ingredient led to a significant reduction in abdominal circumference and thigh circumference and improved skin tone and elasticity.

Key-words

Adipocyte differentiation, adipogenesis, PPAR-γ, lipolytic activity, slenderizing

Introduction

The development of cellulite, still called hydrolipodystrophy, corresponds to the accumulation of fat and reduced local microcirculation in the hypoderm. Cellulite is characterized externally by the appearance of excessive roundness in the hips, abdomen and legs, combined with an « orange skin » tufted appearance of the skin in women (1). Even though felt as bothersome by women, it is a not a disease. To a lesser extent it is even perfectly physiological, a secondary sex character.

Adipose tissue accounts for 15 to 20% of total body weight of a normal individual, equivalent to about 50 to 80 billion fat cells or adipocytes (2). Adipocytes are spherical cells, 40 to 120 µm in diameter that arise from precursor cells, preadipocytes via a process called adipocyte differentiation or adipogenesis.

The capacity of preadipocytes to differentiate into mature adipocytes at the end of adipogenesis is a determining factor for the development of the fat mass. The newly formed mature adipocyte has acquired the capacity to store triglycerides in its lipid vacuole. This is why hyperplasia of adipocytes increases the mass of adipose tissue.

Another well-known mechanism is also responsible for the excessive deposit of fat in adipose tissue: an imbalance between the processes of lipogenesis and lipolysis in differentiated fat cells, leading to hypertrophy of adipocytes.

The present work was focused on the mechanism of adipocyte differentiation, a new approach to the development of slenderizing active ingredients.

Adipocyte differentiation. In the course of adipocyte differentiation, fibroblast-shaped cells (preadipocytes) are transformed to spherical cells. Several steps are required for the transformation of preadipocytes to mature adipocytes, including changes in the expression of a number of genes. This phenomenon is accompanied by considerable modifications in cell morphology, and also of the extracellular matrix.

The process of adipocyte differentiation, or adipogenesis, depends on communication between the cells themselves and between the cells and their environment (3). This involves hormones and varied growth factors that positively or negatively affect adipogenesis.

Several families of transcription factors also participate in the differentiation program. Among them are key molecules such as C/EBPs (CAAT/Enhancer Binding Proteins) or PPARs (Peroxysome Proliferator-Activated Receptors) (4). PPARs are transcription factors belonging to the family of nuclear hormone receptors. Three phenotypes have thus far been described: α, β and γ and each isotype has its own site of expression. The PPAR types preferentially expressed in adipose tissue are isotypes γ. PPAR-γ induces the activation of a number of adipocyte genes and has been identified as one of the major factors in the transcription cascade leading to adipogenesis (5, 6). The expression of PPAR-γ is sufficient to trigger adipogenesis. It also participates actively in the regulation of this phenomenon (3, 7).

Blocking the PPAR-γ mechanism in preadipocytes inhibits the process of adipocyte differentiation (8). The use of a synthetic PPAR-γ antagonist reduces adipogenesis considerably (9).

Until the present, slenderizing strategies have essentially dealt with adipocyte storage capacities by either reducing lipogenesis or increasing lipolysis. Another interesting approach involves inhibiting adipocyte differentiation. In this context, we have developed a slenderizing complex rich in flavonols, obtained from the combination of two plants, *Prunella vulgaris* and *Celosia cristata*. It can both inhibit adipocyte differentiation and stimulate the lipolytic activity of fat cells.

Material and methods

• ***Cell culture***

3T3 F44-2A preadipocytes were inoculated in DMEM (Dulbecco's modified Eagle's medium) (Gibco, Cat. No. 31966) supplemented with 10% donor calf serum (DCS) (Gibco, Cat. No. 16030). The cells were then incubated for 4 days at 37°C in an incubator containing 5% CO_2.

• ***Adipocyte differentiation***

Differentiation of preadipocytes was induced after four days of incubation by replacing the preadipocyte culture medium with DMEM supplemented with 10% fetal calf serum (FCS) (Invitrogen, Cat. No. 10270), 50 nM insulin (Sigma, Cat. No. I-5500), 10^{-6} M biotin (Sigma, Cat. No. B-4639) and 1% (v/v) antibiotics (streptomycin-penicillin) (Gibco, Cat. No. 15070). The cells were then incubated at 37°C in an incubator containing 5% CO_2. The medium was renewed every other day, and daily if there was acidification.

In order to determine the effect of the slenderizing complex on differentiation, the product was added to the culture medium at 0.5%, 1% and 2% (v/v) and to the fresh culture medium added every two days. TNF-α at 0.5 nM (Sigma, Cat. No. T-0157) was used as positive control.

• ***Expression of mRNA coding for PPAR-γ***

After eight days of incubation, the cells were recovered and total RNA extracted. RNA was reverse-transcripted and the complementary DNA obtained was analyzed with the polymerase chain reaction (PCR) technique, using oligonucleotides complementary for a sequence coding for the genes of the protein studied.

The intensity of amplicon bands on agarose gels was quantified by image analysis (BIO-PROFIL® system, BIO-1D software, Vilber-Lourmat, France). The results are expressed as the ratio of intensity of the band of the gene analyzed over that of the internal standard (β-actin).

• ***Oil Red O' staining and quantification of the lipid content***

After the differentiation of preadipocytes into adipocytes, their lipid content was observed under a microscope (Olympus IX 70) after staining with the Oil Red O' method. This technique involves an initial step of fixing cells with 10% formaldehyde (Sigma, Cat. No. F-1635) for 15 minutes, followed by staining cells with a 0.5% (v/v) solution of Oil Red O' (Aldrich, Cat. No. 19819-6) for 1 hour at room temperature.

The lipid content was quantified by adding 5 µl of Adipored reagent (Cambrex-Bioscience, Cat. No. PT-7009) to rinsed cells. The plates were then gently mixed and then incubated for 10 minutes at room temperature. Fluorescence was read with a Fluorilite 1000 fluorimeter (Dynex) with excitation at 485 nm and emission at 572 nm.

• ***Lipolytic activity of mature adipocytes***

After eight 8 days of culture (complete differentiation), the medium was discarded and replaced with DMEM containing 2% FCS and antibiotics but without insulin. The next day, the cells were rinsed with PBS without Ca^{2+} and Mg^{2+} and the medium was replaced with 2 ml of KRBA solution (Krebs-Ringer-Bicarbonate-Albumin) per well. The plates were incubated for 15 minutes at 37°C in an incubator containing 5% CO_2. The slenderizing complex (0.25%, 1% and 1.5%) or caffeine (37.5 g/l at 1%) was added to the corresponding wells and incubated for 120 minutes at 37°C in an incubator containing 5% CO_2.

Non-esterified fatty acids (NEFA) were assayed at 550 nm with the NEFA C colorimetric kit (Wako, Cat. No. FR46551).

• Study of the firming effect in vivo

The aim of this study was to quantify *in vivo* in volunteers the firming effect of the active ingredient formulated at 4% in an emulsion by determining the biomechanical properties of thigh skin with an SEM 575 Cutometer® (Courage-Kazhaka).

The study was conducted on 24 healthy female volunteers (mean age 34 ± 2 years) vs. placebo. The volunteers were selected according to their body mass index (BMI), that had to be included between 21 and 26, as well as by impedance measurement, i.e. having a fat percentage with respect to body weight included between 26% and 35%

• *Study of the slenderizing effect in vivo*

The slenderizing effect of the complex formulated at 4% in an emulsion was determined by measuring abdominal circumference after 56 days of twice daily application.

The study was conducted on 26 healthy female volunteers (mean age 38 ± 2 years) divided into two groups of 13 volunteers each, having applied either the slenderizing complex or the placebo emulsion.

• *Statistical analysis*

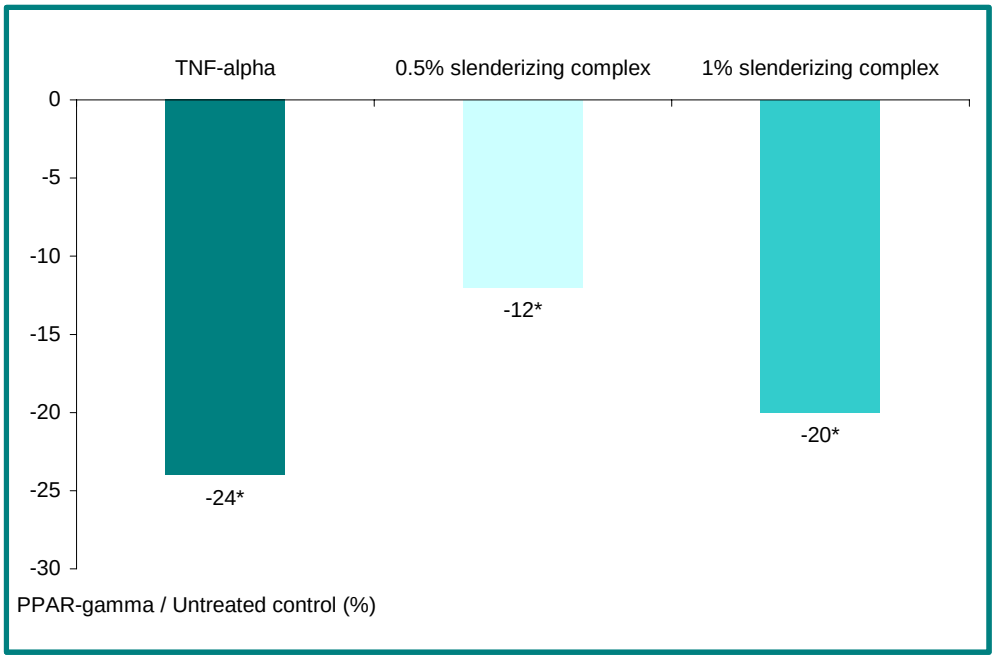
The results were systematically analyzed with Student's test.

Results

• ***The slenderizing complex inhibits adipocyte differentiation***

The capacity of the slenderizing complex to inhibit the process of preadipocyte differentiation into adipocytes was determined by assaying the expression of PPAR-γ. Various concentrations of the slenderizing complex were added to the culture medium during the induction of differentiation. The results were compared to those obtained with TNF-α, a reference molecule known to limit adipocyte differentiation (10).

The addition of 1% slenderizing complex to the preadipocyte culture medium during their differentiation reduced the expression of mRNA coding for PPAR-γ by 20% (figure 1). This effect was comparable to that of TNF-α (-24%). The slenderizing complex thus limits the conversion of preadipocytes into mature adipocytes that can accumulate lipids in their lipid compartment.



**: Significant results according to Student's test (P < 0.05)*

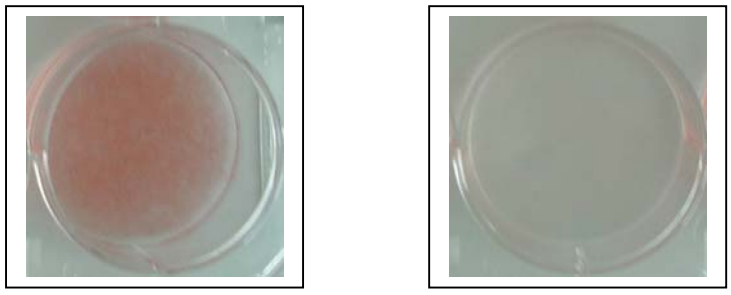
Figure 1: Influence of the slenderizing complex on the expression of mRNA coding for PPAR-γ

• ***<u>The slenderizing complex reduces the quantity of lipids accumulated during adipocyte differentiation</u>***

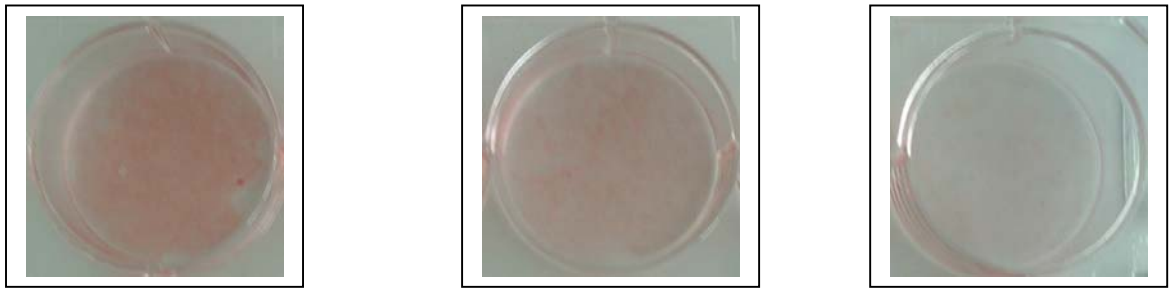
The effect of the slenderizing complex on adipocyte differentiation was confirmed by quantifying the intracellular lipid content of differentiated adipocytes with the Oil Red O' staining method. The intracellular lipid content of untreated control cells after staining exhibited a large quantity of stained lipid droplets characteristic of mature adipocytes.

After six days of incubation with 0.5 nM TNF-α, the positive control known to inhibit adipocyte differentiation, the cells remained undifferentiated, remaining in the preadipocyte stage: few lipid droplets were observed (figure 2). Quantification of the intracellular lipid content by fluorimetric assay revealed that it content was reduced by 71% in mature adipocytes arising from preadipocytes treated with TNF-α compared to untreated preadipocytes.

After incubation with 2% slenderizing complex, the quantity of lipid droplets was reduced by 65% (figure 2). This effect was thus comparable to that of TNF-α. In the absence of lipolysis, the slenderizing complex reduces the intracellular lipid content that in fact corresponds to the inhibition of the differentiation process of preadipocytes into mature adipocytes.



Untreated control (+++++) 0,5 nM TNF-α (+)



0.5% slenderizing complex (++++) 1% slenderizing complex (+++) 2% slenderizing complex (++)

Highly numerous lipid droplets: +++++

Rare lipid droplets: +

Figure 2: Effect of the slenderizing complex on adipocyte differentiation.

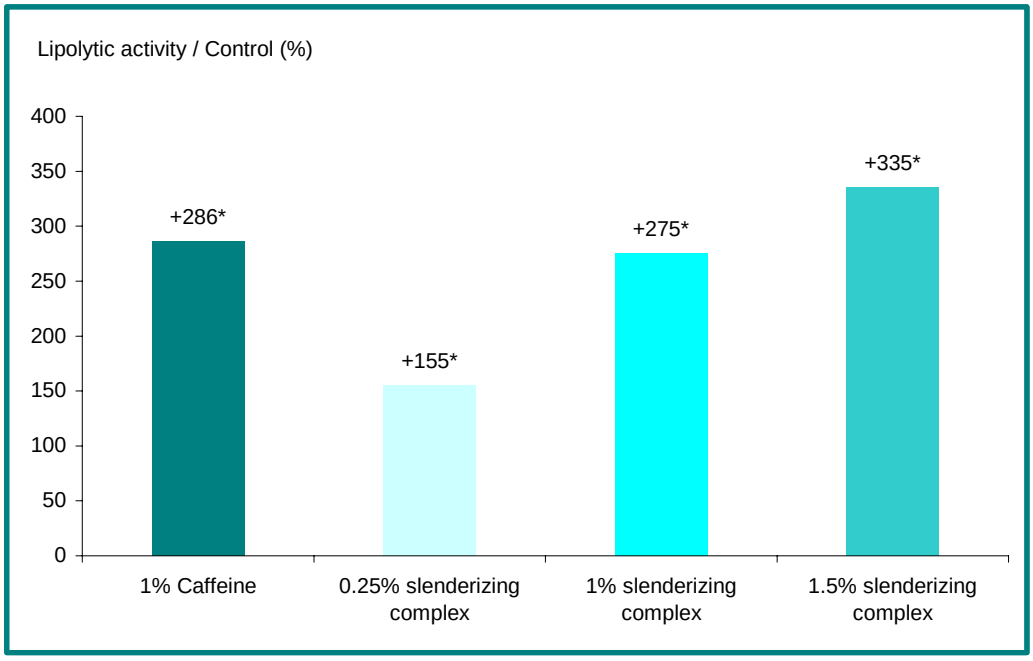
Observation of lipid contents.

• ***The slenderizing complex stimulates the lipolytic activity of adipocytes***

Lipolysis is the step that mobilizes fat by the transformation of triglycerides stored in mature adipocytes into free fatty acids and glycerol. Caffeine, known to increase the concentration of cAMP and thus to stimulate lipolysis, was used as positive control in this experiment. The addition of 1% caffeine to the culture medium favored the lipolytic activity of fat cells by 286% (figure 3).

Tested at 1%, the slenderizing complex significantly increased the lipolytic activity of fat cells by 335% (figure 3).

In parallel to its inhibition of adipocyte differentiation, the slenderizing complex also stimulated the transformation of triglycerides in the lipid compartment of adipocytes into free fatty acids and glycerol.



**: Significant results according to Student's test (P < 0.05)*

Figure 3: Influence of the slenderizing complex on the lipolytic activity of mature adipocytes. Comparison to caffeine.

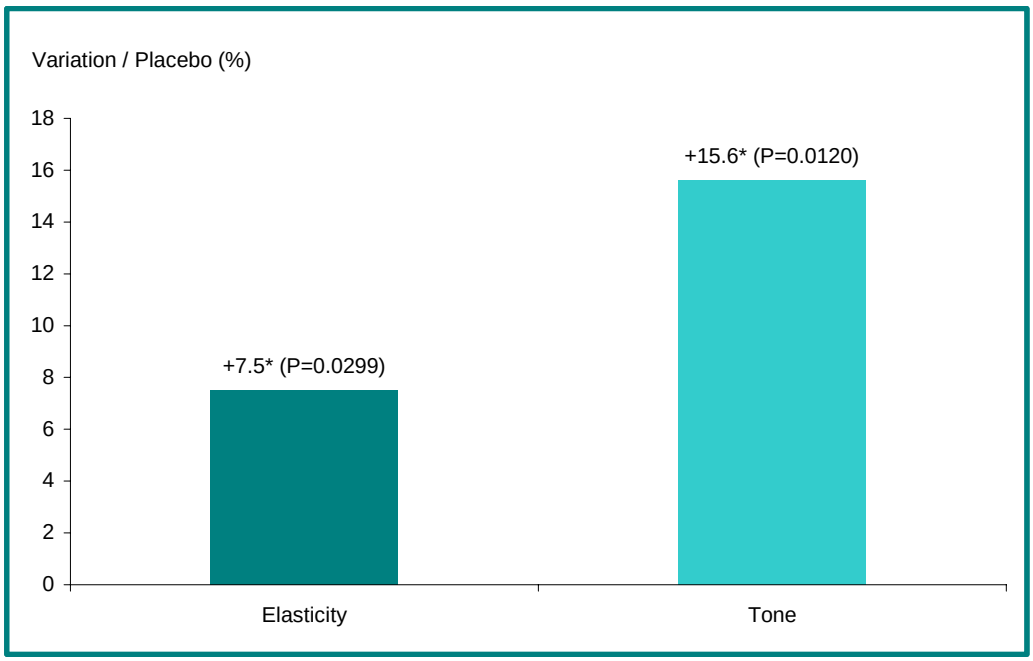
• ***The slenderizing complex improves skin tone***

The aim of this study was to quantify *in vivo* in volunteers the firming effect of the slenderizing complex formulated at 4% in an emulsion by determining the biomechanical properties of thigh skin with a Cutometer®.

After 28 days of twice daily application and in comparison to the placebo, the slenderizing complex significantly improved the following parameters (figure 4):

- Ur/Uf that represents skin elasticity (7.5%, P = 0.0299).
- Ur that represents skin tone (15.6%, P = 0.0120).

This effect was observed in 71% of the volunteers.



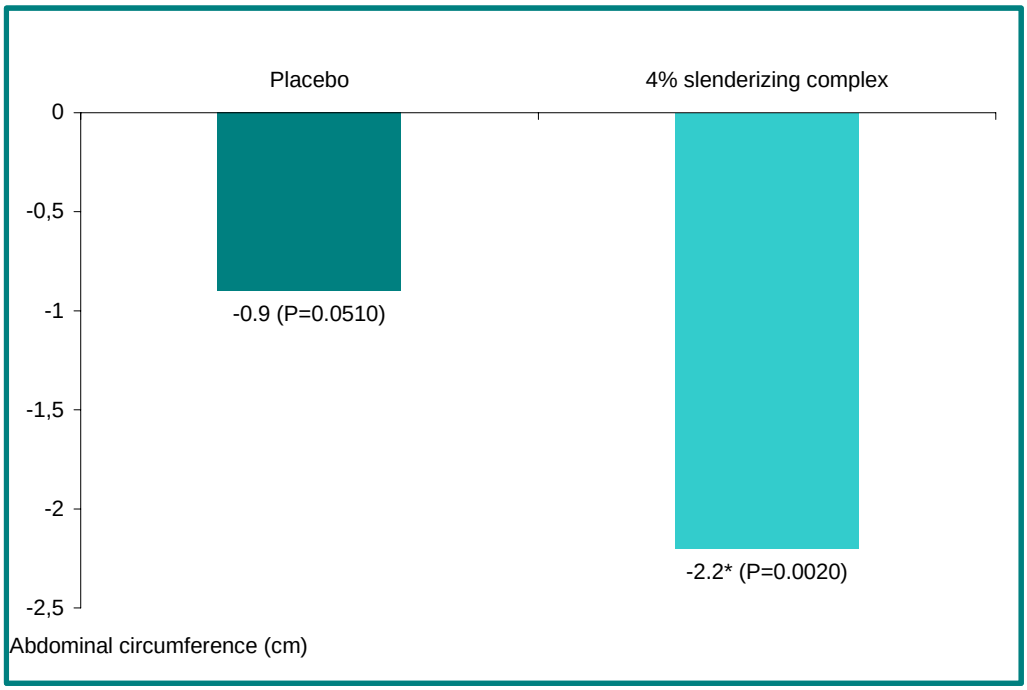
**: Significant results according to Student's test (P < 0.05)*

Figure 4: Firming effect of the slenderizing complex after 28 days of treatment. Comparison to the placebo formula.

• ***The slenderizing complex favors a reduction in abdominal circumference***

The slenderizing effect of the active ingredient was investigated by measuring the abdominal circumference of 26 female volunteers distributed in two groups of 13 volunteers each, one applying the placebo emulsion, the other the slenderizing complex formulated at 4% in an emulsion. After 56 days of twice daily application, the volunteers testing the placebo tended to lose an average of 0.9 cm of abdominal circumference without losing weight. The maximal loss observed was 3.8 cm (figure 5).

In the same conditions, the volunteers testing the slenderizing complex lost an average of 2.2 cm of abdominal circumference without losing weight (the maximal reduction observed with the slenderizing complex was 5.7 cm). This variation was significant according to Student's test and was observed in 77% of the volunteers.



**: Significant result according to Student's test (P < 0.05)*

Figure 5: Variation of abdominal circumference after 56 days of treatment with the slenderizing complex. Comparison to the placebo formula.

Conclusion

Until the present time, slenderizing strategies dealt with the lipolysis/lipogenesis balance. Their aim was to favor lipolysis by increasing the concentration or activity of cyclic AMP or to inhibit lipogenesis by limiting the transport of fatty acids.

The better understanding of molecular and cellular phenomena involved in the growth of adipose tissue has enabled innovating approaches to be developed to limit the storage of fat in adipocytes.

We have shown in the present work that an active ingredient obtained from *Celosia cristata* and *Prunella vulgaris* tested at 1.5% stimulates the lipolytic activity of mature adipocytes (+335%). In addition, it limits the differentiation of preadipocytes into adipocytes by reducing (-20%) the expression of mRNA coding for PPAR-γ comparably to the positive control TNF-α. This is a key transcription factor without which the differentiation of preadipocytes cannot occur.

The inhibition of adipocyte differentiation was confirmed by the microscopic observation of a reduction in the lipid content of newly formed adipocytes. This finding confirms that only a limited number of preadipocytes could differentiate completely into mature adipocytes in the presence of the slenderizing complex.

Finally, the slenderizing complex was tested directly *in vivo*. Formulated at 4% in an emulsion, it significantly improved skin tone (+15.6%) and elasticity (+7.5%) after one month of use. After 56 days of treatment, it also led to a significant reduction in abdominal circumference, 2.2 cm on average, and also thus favored a decrease in thigh circumference.

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