In vitro and clinical efficacy of the use of phytoestrogens-based topical cosmetic use in photoaged skin

Eficácia in vitro e clínica do uso cosmético tópico de fitoestrógenos em pele fotoenvelhecida

ABSTRACT

Introdução: Introduction: Skin aging is a challenge to treat.

Objectives: To evaluate the in vitro and in vivo efficacy and clinical safety of a phytoestrogens-based
cosmetic in the management of photoaging.

Methods: The in vitro investigation was performed using the analysis of the genic expression of fibronectin and procollagen, evaluation of the immunomodulatory activity (proinflammatory and anti-inflammatory cytokines synthesis) and histochemistry and immunofluorescence analyses of the skin and the dermoepidermal junction. The in vivo investigation – performed in 76 women randomized into Group A (phytoestrogens complex cream and SPF 20 twice daily) or Group B (the same product plus a commercially available anti-aging cream applied overnight). The study lasted for 120 days, with physician- and patient-led evaluations,
in addition to monthly ultrasound (20 MHz) and photographic analysis. Skin biopsies of the face were performed before and after treatment.

Results: The study showed In vitro: increase in the expression of fibronectin, in procollagen, immunomodulator potential, represented by an increase in IL-1α and a decrease in IL-10; improvement in the integrity of the dermoepidermal junction; increase in the viability and thickness of the epidermis; increase in collagen synthesis .In vivo: subjective global improvement of the skin's appearance; reduction in the count and intensity of spots, erythema, skin pores, and cutaneous porphyrin. The ultrasound and biopsy
revealed increased dermal density (52.7%) and dermal fibers (22.3%), respectively.

Conclusions: The topical use of phytoestrogens-based cosmetics improves the overall condition of the skin.

Keywords: collagen, photoaging, skin aging.

RESUMO

Introdução: *O tratamento do envelhecimento cutâneo representa um desafio clínico.*

Objetivos: *Avaliar a eficácia in vitro e in vivo, e a segurança clínica de cosmético com fitoestrógenos na abordagem do fotoenvelhecimento.*

Métodos: *A etapa in vitro foi realizada pela análise da expressão gênica
de fibronectina e pró-colágeno, avaliação da atividade imunomoduladora e análise histoquímica e por imunofluorescência da pele e da junção dermoepidérmica com o produto analisado. No estudo clínico in vivo foi 76 mulheres, foram randomizadas em dois grupos: o Grupo A usou creme contendo complexo de fitoestrógenos e FPS 20 duas vezes ao dia, enquanto o Grupo B usou este mesmo creme associado a outro com função de antienvelhecimento aplicado à noite. O estudo durou 120 dias tendo sido realizadas mensalmente avaliações médicas, da voluntária, ultrassonografia (20MHz), fotografias e biópsias pré e pós-tratamento.*

Resultados: *No estudo in vitro houve aumento na expressão de fibronectina e procolágeno, potencial imunomodulador, representado pelo aumento de IL-1α diminuição de
IL-10; melhora da integridade da JDE, aumento da viabilidade e espessura da epiderme, e da síntese de colágeno. in vivo: melhora global subjetiva da aparênciada pele da face; redução de manchas, eritema, poros e porfirina cutânea. O ultrassom e a biópsia revelaram aumento da densidade dérmica (52,7%) e de fibras dérmicas (22,25%), respectivamente.*

Conclusões: *Fitoestrógenos tópicos melhoram a condição geral da pele, avaliada clínicamente, histológicamente e por ultrassonografia; acrescentam-se resultados in vitro de aumento da síntese de fibronectina, prócolágeno e colágeno, melhoria da integridade da junção dermoepidérmica e restauração da resposta imunológica da pele.*

Palavras-chave: *colágeno, fotoenvelhecimento, envelhecimento da pele.*

Original Article

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INTRODUCTION

Skin aging is a degenerative, insidious, complex, and multifactorial process (notably caused by UV irradiation) that will unavoidably affect all human beings. [1] Heavy exposure of the skin to external aggression associated with genetic, metabolic, endocrine, immunological, and other intrinsic elements makes the susceptibility of the cutaneous tissue events related to aging more marked and more visually prominent. [2]

Skin aging is caused by two concurrent processes: intrinsic and chronological aging – when it affects areas that are protected from the sun – and extrinsic aging or photoaging – when it affects areas exposed to the sun. [3,4]

Intrinsic skin aging is determined by genetic factors and aggravated by neurohormonal factors, and is thus independent of external or environmental factors. [1,4,5] Although it is affected by the same degeneration mechanisms that are seen in other organs, the skin is one of the organs that suffers the most from aging. [1,5] Hormonal changes that take place as a part of aging will occur in direct correlation with the skin's phenotype (phototype). [5] In areas protected from the sun – which are predominantly affected by intrinsic aging – the skin becomes thinner, there is an emergence of delicate wrinkles and roughness, and a loss of elasticity and subcutaneous fat; however it is a milder process than photoaging. [1] In intrinsic aging, a reduction in the number and function of fibroblasts and the destruction of important structures – especially collagen, elastin, and fibronectin – can be observed, together with an alteration in proliferative cellular homeostasis, which often results in malignant or irreversible lesions. [6,7]

Collagen fibers confer structural and mechanical integrity to the dermis, and elastin plays an important role in the skin's elasticity. 8 Fibronectin is capable of contracting and organizing the connective tissue, promoting cell adhesion during the healing process, and promoting re-epithelialization – and it is the main factor responsible for the integrity of the dermoepidermal junction (DEJ). [9-11] With aging, there is significant reduction in the quantity and quality of fibronectin observed in the DEJ, which corresponds to one of the main markers of dermoepidermal aging. [10,12] Rocquet and colleagues demonstrated that decreased amounts of fibronectin are found in wrinkles, and that its enzymatic degradation significantly increases with age. [11]

The immune system's efficiency decreases dramatically as individuals age, which is a major cause of the physical appearance of skin aging and of the susceptibility to infections and cancer. [13] The reason for the degradation of the immune system is still unclear, nonetheless the relevance of the reduction in the number of Langerhans cells in the skin, of the defect in memory of T cells, of the decrease in the proliferative response of lymphocytes and of the reduction of the body's ability to produce antibodies, is well known. [13,14] Aging alters the pattern of the skin's immune system – which changes from a Th1 (T helper 1) response pattern to a dominant Th2 pattern. The Th1 response – with IL-1 (interleukin 1), IL-8, TNF (tumor necrosis factor alpha), INF (interferon gamma), adhesion molecules, chemokines, eicosanoids, and nitric oxide – triggers the physiological events that culminate in tissue degradation, since that pattern produces proinflammatory cytokines. The Th2 immune pattern is accompanied by an increase in IL-4, IL-5, and IL-10; IL-10 contains the inflammatory response, considerably favoring the acceleration of intrinsic aging. [13 14]

Extrinsic aging (or photoaging) is characterized by the total effects of continuous exposure to environmental factors such as solar radiation, temperature, mechanical energy, changes in humidity, and/or chemical or biological contaminants. [2] Photoaging results from UV irradiation, which mainly damages the skin's morphological dermal structures and affects its consistency and resilience, causing early photoaging. [4,15] Extrinsic aging is a cumulative process that occurs based on the patient's phototype and degree of sun exposure. [5]

Ultraviolet (UV) and infrared radiation causes alterations in cellular components and activates the matrix metalloproteinases, which changes the collagen extracellular matrix and degrades its integrity – thus causing alterations, mainly in the dermis. [15,16] UV irradiation also affects epidermal structures, keratinocytes, and fibroblasts, which in turn activates surface receptors that transmit a signal capable of causing molecular changes that lead to the destruction of extracellular collagen and halt the synthesis of new collagen, and cause the disorganized accumulation of elastin and its component, the fibrin, in the deep dermis, in addition to a considerable loss of interstitial collagen. [17]

This irradiation also leads to the formation of pathogenic agents that produce free radicals (reactive oxygen species, ROS), which play a crucial role in the degradation and damage of the skin's defensive non-enzymatic and enzymatic antioxidant systems. [3,4,17] They damage the noble structures of the skin, such as cell membranes, DNA segments, and collagen and elastic fibers, thus causing cutaneous aging. [1,3,6,17] As a result, the skin exposed to UV irradiation has a more coarse and dry appearance, with deep and well-demarcated wrinkles and speckled pigmentation. 15 Infrared radiation is also involved in photoaging and photodamage (carcinogenesis). [16]

In light of these circumstances, this study explored the possible contribution of a cosmeceutical product based on phytoestrogens in the improvement and prevention of aesthetic manifestations of aging by observing dermoepidermal histological alterations.

METHODS

This in vitro and in vivo study was approved by the university's research ethics committees. The in vitro stage was carried out using three methods of analysis. The studies comprised the use of human cells under optimal culture conditions, was and were carried out in accordance with current methodologies and applied, accepted and validated by the international scientific community.

The first method was carried out in vitro in order to allow the observation of how the phytoestrogen-based product behaved regarding the gene expression of fibronectin and type I procollagen. Human keratinocytes (Cascade Biologics, Inc. –

Portland, OR, USA) and fibroblast (Lonza Walkersville, Walkersville, USA) cultures were carried out in specific culture media. Both were seeded in 75 cm2 bottles, cultured and expanded in wet ovens at 37°C in the presence of 5% CO_2. The incubation time for keratinocytes and fibronectin was six hours. For pro-collagen and fibroblasts it was 12 hours. Cell viability was determined using the MMT technique ((3 - (4.5 dimethylthiazol-2yl) – 2.5- tetrazoline diphenyl bromide). Real-time polymerase chain reaction (PCR) was used to assess the gene expression of fibronectin and pro-collagen, with results calculated based on the amount of mRNA.

The second method comprised the evaluation of the immunomodulatory activity of the cosmetic's active principle (proinflammatory and anti-inflammatory cytokines syntheses). The analysis was carried out by isolating the phytoestrogen – which is a stabilized extract of three red and brown seaweeds – and subsequent analysis was conducted of their effects on the production of pro-inflammatory (Th1) IL-1 and anti-inflammatory (Th2) IL-10 cytokines in cultured human keratinocytes. These keratinocytes were seeded, cultured, and expanded in a wet oven at 37°C. The cultures were incubated with six non-cytotoxic concentrations of the product, previously determined by the MMT technique. The cells were kept in contact with the test product and lipopolysaccharide (LPS), which is used to chronically stimulate cells in order to simulate chronological or micro-inflammatory aging, and then the potential immunomodulatory activity of the test product was evaluated in vitro for three consecutive days, with the subsequent collection of the supernatant. The cytokines were quantified using immunoenzymatic trial kits (Elisa), and the capture monoclonal antibody anti-cytokine was added to the plate.

The third method consisted of histochemical and immunofluorescence analysis of the skin and DEJ using the study product. The general characteristics of the skin – such as the stratum corneum conditions, the viable epidermis, and the number of microvilli – were evaluated, and the marking of the fibronectin. The analysis was carried out using immunofluorescence based on the incubation of the product in ex vivo skin fragments with primary antibody anti-fibronectin and afterwards with Alexa Flour. The histochemical analysis was carried out with hematoxylin-eosin (HE) staining. Histologic cuts were also stained with Sirius Red for the visualization and analysis of collagen fibers.

The in vivo phase was a 120-day prospective, open, monocentric, phase IV, comparative, randomized clinical study involving 76 volunteers who were randomized into two groups of 38 individuals (aged 45-70, phototypes I to III). The volunteers were included after having read and signed a term of free and informed consent. The study was conducted in accordance with the principles of the Declaration of Helsinki, Good Clinical Practices, and the International Conference of Harmonization guidelines.

The volunteers underwent the wash-out with the isolated use of SPF 15 sunscreen (Episol® SPF 15, Mantecorp Indústria Química e Farmacêutica Ltda., Rio de Janeiro/RJ, Brazil) for 30 days, used twice daily (morning and lunchtime). The study product was then dispensed to each group:

Group A) phytoestrogens-based anti-aging product + SPF 20 (Age Care FPS 20 e PPD 10, Mantecorp Indústria Química e Farmacêutica Ltda., Rio de Janeiro/RJ, Brazil) in the morning and at lunchtime.

Group B) product similar to that of Group A, with the same posology, combined with a different commercial anti-aging cosmeceutical product (Epidrat Lift, Mantecorp Indústria Química e Farmacêutica Ltda., Rio de Janeiro/RJ, Brazil), used at night. The volunteers used the study products for 90 consecutive days.

In preparation for the screening process, the volunteers were checked regarding all inclusion criteria (aged 45-70; menopausal; Fitzpatrick phototype I to III; free of diseases that, according to the evaluator physician, might interfere with the assessment of skin aging; trained and able to join and follow the scheme of visits and treatment; absence of known history of allergic reaction to the test product's components; use of SPF 15 facial sunscreen for at least 30 days prior to baseline) and all exclusion criteria (use of medications, cosmetics, or treatments that, according to the evaluator physician, could interfere in the assessment of the response being studied; any other reason that, according to the evaluator physician's discretion, could place the volunteer at risk or interfere with the study's objectives; intense sun exposure during the 60 days prior to screening; presence of skin lesions in the area assessed; abuse of illicit drugs; smoking; endocrine diseases, in particular gonadal, suprarenal, and/or thyroidal).

Both the exclusion criteria and the voluntary adhesion were evaluated every 30 days (participants were instructed not to interrupt the use of products for more than five successive days or 10 total days, throughout the study period). In addition, the patients underwent 20 MHz ultrasound (USB-SkinScanner DUB6100, Taberna Pro-TPM Medicum GmbH, Lüneburg, Germany) in the skin. Adverse events were evaluated, and photographic analysis was performed (Canon TM Power Shot G10, Japan). The physician evaluator and the volunteers also subjectively assessed tolerability to the product and the therapeutic response.

The subjective evaluation of efficacy was carried out using a scale to rate the patients' answers (+4: total improvement; +3: marked improvement, +2: moderate improvement, +1: slight improvement; 0: unchanged; -1: slight worsening, -2, moderate worsening, -3, marked worsening; -4: total worsening). Criteria were used in the subjective assessment of tolerability (Excellent: total absence of adverse events; Good: easily tolerated events; Moderate: tolerable events that did not lead to the discontinuation of treatment; Severe: events that required the discontinuation of treatment).

Dermal density was evaluated using ultrasonography, followed by a subjective and comparative analysis with the previous ultrasound image, according to a rating scale (increased considerably; increased; unchanged; decreased; decreased considerably). In addition, a skin biopsy was carried out in the face (preauri-

cular region) with a n.2 punch in order to assess the pattern of collagen fibers (Masson's Trichrome) and elastic fibers (Verhoeff).

The statistical test to assess the equality of two proportions was conducted to evaluate the results obtained from the application of the evaluator physician's and patients' questionnaires, and the results of the ultrasound and skin biopsies. That test compares the proportion and ratings of the answers to two specific variables to determine whether they are statistically significant. Throughout the study, results were considered statistically significant a $p < 0.05$.

In the in vitro stage of the immunomodulatory activity, the analysis of variance (ANOVA) statistical technique was used. The Tukey's test was used when the ANOVA detected significant differences between the groups. For all studied groups, those with $p < 0.05$ were considered statistically significant. In the gene expression of fibronectin and procollagen stages, the expressions were considered relevant (or significant) when the values obtained were 1.5 greater than that of the control. On the other hand, inhibition of expression was considered relevant when the values obtained were 0.5 less than that of the control.

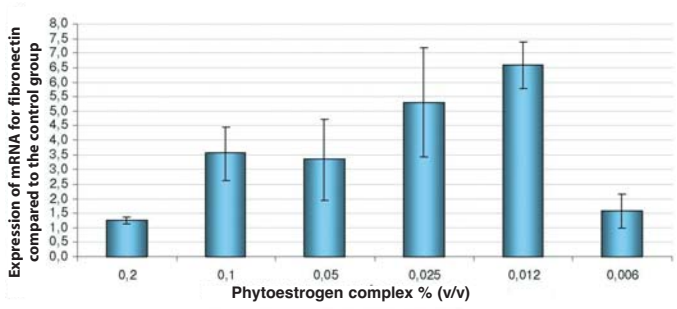
RESULTS

The results obtained in the in vitro analysis demonstrated significant changes in the evaluation of the product. Regarding the stage in which the gene expression of fibronectin and type I procollagen were evaluated, it was considered that a significant increase had occurred when the values were 1.5 greater than that of the control. It was considered that there had been relevant inhibition of expression when values were 0.5 lesser than that of the control. The incubation of the complex of phytoestrogens in human keratinocytes cultures was capable of producing a significant increase in the relative expression of fibronectin (in the form of mRNA) in the concentrations of 0.1, 0.05, 0.025, and 0.012% (Graph 1). Regarding the relative expression of type I procollagen (also in the form of mRNA), the phytoestrogens complex was capable of significantly increasing its relative expression in the concentrations of 0.2, 0.1, and 0.05% (Graph 2).

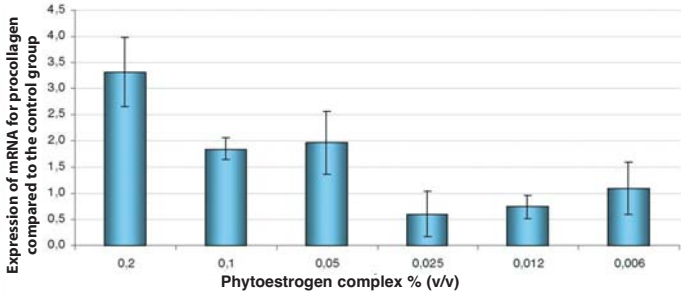
Regarding the assessment of immunomodulatory activity, we observed that the chronic incubation of cells with LPS in fact simulates chronological aging, generating alterations in the immune response through increased IL-10 (+ 3.68 times) and a slight reduction of IL-1 (-1.8 times). However, when the phytoestrogens complex was added to the cell cultures that were chronically incubated with LPS, the response's profile was changed, making the baseline levels of the proinflammatory cytokine IL-1 increase and return to control levels. The complex reduced IL-10 levels in the concentrations of 1.6%, 0.8%, 0.4%, and 0.2% by approximately three times as compared to the control group, which received LPS only (Graphs 3 and 4).

In the immunofluorescence analysis, there was a clear increase in the anti-fibronectin signal's fluorescence intensity in the DEJ (Figure 1).

An improvement in the general conditions of the fragments treated with the phytoestrogen complex could be verified with the HE staining technique (Figure 2). The comparison with the control showed an increase in the viability and thickness of the epidermis, greater cohesion and compacting of the stratum corneum, and an increase in the DEJ's microvilli. The visualization of collagen fibers using the Sirius Red staining demonstrated greater intensity and uniformity of the red color (collagen fibers) compared to the controls (Figure 3).



Graph 1: Relative expression of mRNA for fibronectin in cultured human keratinocytes incubated with the phytoestrogen complex



Graph 2: Relative expression of mRNA for procollagen in cultured human keratinocytes incubated with the phytoestrogen complex

Once the in vitro stage had been completed, the study entered the in vivo stage. Of the 76 volunteers, 72 completed the study and four quit for personal reasons, unrelated to the test products.

The volunteers' subjective assessment of effectiveness suggested good performance in both groups, in particular regarding the improvement of wrinkles, thin lines, melanoses, other hyperchromias, hydration, vitality, softness, and overall appearance. The comparative analysis between groups did not reveal many differences, except for erythema – which was associated with an increase in the response "moderate improvement" in Group B, and an increase in the response "unchanged" in Group A (Table 1).

The physician's subjective assessment of efficacy suggested, in general, that there were good results in both groups. Again, there were few between-group differences. Group B presented better performance than Group A only at the D90 visit for erythema and thin lines (Table 2).

The groups presented good cutaneous tolerability, as illustrated, with no statistical differences in either the visit-to-visit

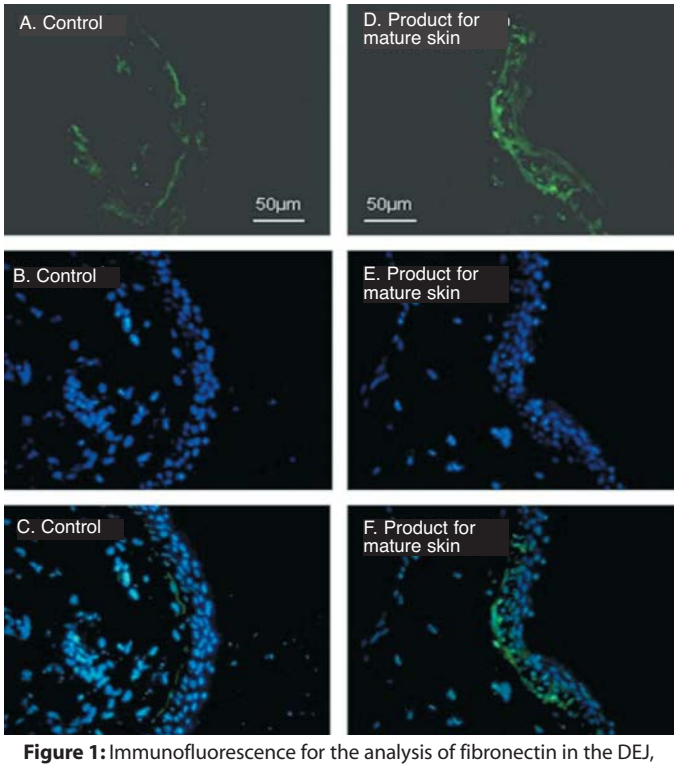


Figure 1: Immunofluorescence for the analysis of fibronectin in the DEJ, to evaluate its response to the phytoestrogens complex *in vitro*

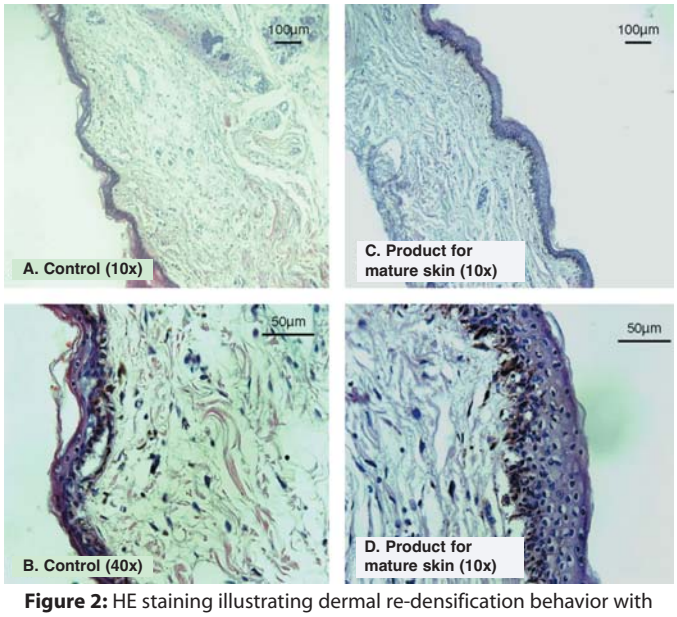


Figure 2: HE staining illustrating dermal re-densification behavior with *in vitro* use of phytoestrogens complex

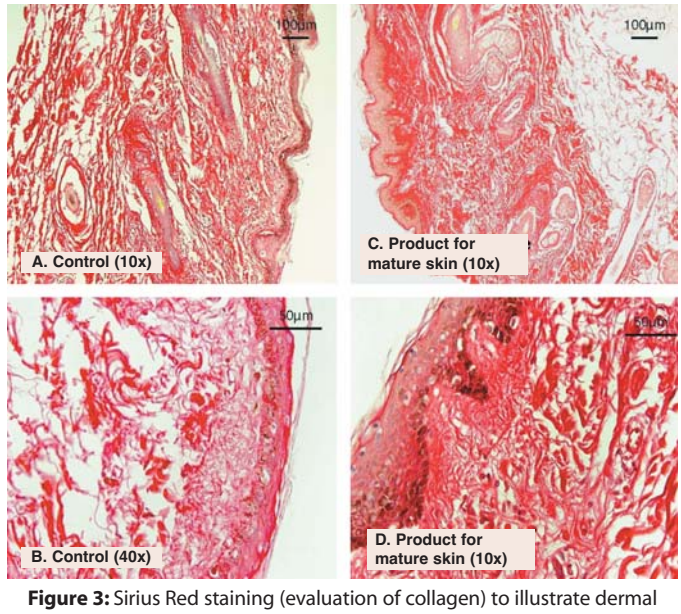


Figure 3: Sirius Red staining (evaluation of collagen) to illustrate dermal re-densification behavior with *in vitro* use of phytoestrogens complex

evaluation within each group or the comparative evaluation between groups. Remarkably, the most common response was the "absence" of adverse events for all parameters of the subjective evaluation of tolerability (erythema, dryness and squamation) (Table 3). Regarding the assessment of product safety, there were no statistically significant differences between visits for both groups regarding the adverse events described. There were no adverse effects in Group A, while in Group B there was only one episode of erythema in the malar region (in the nasal ala) on D60, which was probably caused by the use of the products. The erythema resolved completely and spontaneously prior to D90, without the interruption of use or voluntary exclusion from the clinical trial. Regarding adverse events not related to the use of the products, all were resolved in both groups.

In the ultrasound evaluation, both groups presented a favorable response regarding dermal re-densification – in both groups, there was prevalence of the responses "increased considerably" and "increased" for the amount of collagen from baseline to D30. When comparing the groups, Group A presented a decrease in the response "increased considerably" on the D60 visit, and the other answers remained unchanged compared to D0. On D90, there was a decrease in the answer "increased" and an increase in the responses "decreased" and "decreased considerably" compared to D60. In Group B there was a prevalence of the answer "unchanged" and an increase in the answer "decreased" on D60 compared to D30. On D90 there was a significant increase in the response "increased" and a decrease in the responses "unchanged" and "decreased" in relation to D60, thus indicating a recovery in the performance with the use of Group B products (Table 4, Figure 4).

Based on the histological analysis of skin biopsies, an increase in the amount of collagen fibers was observed in 27.80% and 19.40% of the volunteers in Group A and Group B, respectively, with a statistically significant between-group comparison ($p = 0.405$). There was an increase of 16.70% in elastic fibers for Groups A and B. Regarding mucin, Group B presented an increase in 2.80% of patients, with a statistically significant between-group comparison ($p = 0.314$) (Table 5, Figure 5).

Table 1: Volunteers' subjective criteria for clinical analysis before and after 90 days of using product based on phytoestrogens. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

	Group A					Group B					Statistical significance for comparison between groups	
	D30		D90		Statistical significance Group A	D30		D90		Statistical significance Group B	D30	D90
	N.	%	N.	%		N	%	N	%			
Wrinkles												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	12	33	4	11	0,023	6	17	3	8	0,285	0,102	0,691
Slight improvement	10	28	13	36	0,448	14	39	9	25	0,206	0,317	0,306
Moderate improvement	13	36	11	31	0,617	9	25	12	33	0,437	0,306	0,800
Marked improvement	1	3	8	22	0,013	7	19	12	33	0,181	0,024	0,293
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Thin lines												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	12	33	3	8	0,009	8	22	4	11	0,206	0,293	0,691
Slight improvement	8	22	14	39	0,125	12	33	6	17	0,102	0,293	0,035
Moderate improvement	15	42	10	28	0,216	9	25	12	33	0,437	0,134	0,609
Marked improvement	1	3	9	25	0,006	7	19	14	39	0,070	0,024	0,206
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Solar melanoses												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	10	28	6	17	0,257	9	25	10	28	0,789	0,789	0,257
Slight improvement	12	33	10	28	0,609	8	22	5	14	0,358	0,293	0,147
Moderate improvement	11	31	6	17	0,165	15	42	7	19	0,041	0,326	0,759
Marked improvement	3	8	14	39	0,002	3	8	13	36	0,005	1,000	0,808
Total improvement	-	-	-	-	-	1	3	1	3	1,000	0,314	0,314
Other hyperchromias												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	10	28	7	19	0,405	9	25	10	28	0,789	0,789	0,405
Slight improvement	12	33	9	25	0,437	8	22	3	8	0,101	0,293	0,058
Moderate improvement	11	31	6	17	0,165	15	42	9	25	0,134	0,326	0,384
Marked improvement	3	8	14	39	0,002	3	8	14	39	0,002	1,000	1,000
Total improvement	-	-	-	-	-	1	3	-	-	0,314	0,314	-
Erythema												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	24	67	26	72	0,609	24	67	11	31	0,002	1,000	<0,001
Slight improvement	6	17	4	11	0,496	4	11	3	8	0,691	0,496	0,691
Moderate improvement	2	6	3	8	0,643	6	17	13	36	0,061	0,134	0,005
Marked improvement	4	11	3	8	0,691	2	6	9	25	0,022	0,394	0,058
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Hydration												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-

Continue

Table 1: Volunteers' subjective criteria for clinical analysis before and after 90 days of using product based on phytoestrogens. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

	Group A					Group B					Statistical significance for comparison between groups	
	D30		D90		Statistical significance Group A	D30		D90		Statistical significance Group B	D30	D90
	N.	%	N.	%		N	%	N	%			
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	1	3	4	11	0,164	-	-	-	-	-	0,314	0,040
Slight improvement	3	8	1	3	0,303	-	-	-	-	-	0,077	0,314
Moderate improvement	14	39	6	17	0,035	11	31	5	14	0,089	0,458	0,743
Marked improvement	17	47	25	69	0,056	24	67	27	75	0,437	0,096	0,599
Total improvement	1	3	-	-	0,314	1	3	4	11	0,164	1,000	0,040
Vitality												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	5	14	2	6	0,233	1	3	-	-	0,314	0,088	0,151
Slight improvement	3	8	7	19	0,173	-	-	3	8	0,077	0,077	0,173
Moderate improvement	12	33	13	36	0,804	16	44	17	47	0,813	0,334	0,339
Marked improvement	16	44	14	39	0,633	19	53	15	42	0,345	0,479	0,810
Total improvement	-	-	-	-	-	-	-	1	3	0,314	-	0,314
Oleosity												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	1	3	0,314	-	0,314
Slight worsening	-	-	-	-	-	1	3	-	-	0,314	0,314	-
Unchanged	20	56	22	61	0,633	9	25	23	64	0,001	0,008	0,808
Slight improvement	7	19	7	19	1,000	6	17	1	3	0,047	0,759	0,024
Moderate improvement	4	11	4	11	1,000	12	33	5	14	0,052	0,023	0,722
Marked improvement	3	8	3	8	1,000	8	22	6	17	0,551	0,101	0,285
Total improvement	2	6	-	-	0,151	-	-	-	-	-	0,151	-
Smoothness												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	-	-	1	3	0,314	-	-	-	-	-	-	0,314
Slight improvement	2	6	5	14	0,233	4	11	1	3	0,164	0,394	0,088
Moderate improvement	13	36	7	19	0,114	10	28	4	11	0,074	0,448	0,326
Marked improvement	20	56	23	64	0,471	21	58	30	83	0,020	0,812	0,061
Total improvement	1	3	-	-	0,314	1	3	1	3	1,000	1,000	0,314
Overall appearance												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	1	3	2	6	0,555	-	-	-	-	-	0,314	0,151
Slight improvement	4	11	2	6	0,394	1	3	3	8	0,303	0,164	0,643
Moderate improvement	15	42	11	31	0,326	15	42	12	33	0,465	1,000	0,800
Marked improvement	16	44	21	58	0,238	20	56	21	58	0,812	0,346	1,000
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-

Table 2: Evaluator physician's subjective criteria for clinical analysis before and after 90 days of using phytoestrogens-based product in volunteers with signs of photoaging. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

	Group A					Group B					Statistical significance for comparison between groups	
	D30		D90		Statistical significance Group A	D30		D90		Statistical significance Group B	D30	D90
	N.	%	N.	%		N	%	N	%			
Wrinkles												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	13	36	4	11	0,013	13	36	4	11	0,013	1,000	1,000
Slight improvement	14	39	30	83	<0,001	18	50	28	78	0,014	0,343	0,551
Moderate improvement	9	25	2	6	0,022	5	14	4	11	0,722	0,234	0,394
Marked improvement	-	-	-	-	-	-	-	-	-	-	-	-
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Thin lines												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	8	22	3	8	0,101	10	28	2	6	0,011	0,586	0,643
Slight improvement	16	44	27	75	0,008	21	58	13	36	0,059	0,238	0,001
Moderate improvement	12	33	6	17	0,102	5	14	21	58	<0,001	0,052	<0,001
Marked improvement	-	-	-	-	-	-	-	-	-	-	-	-
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Solar melanoses												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	8	22	5	14	0,358	10	28	6	17	0,257	0,586	0,743
Slight improvement	16	44	16	44	1,000	22	61	16	44	0,157	0,157	1,000
Moderate improvement	12	33	15	42	0,465	3	8	9	25	0,058	0,009	0,134
Marked improvement	-	-	-	-	-	1	3	4	11	0,164	0,314	0,040
Total improvement	-	-	-	-	-	-	-	1	3	0,314	-	0,314
Other hyperchromias												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	11	31	5	14	0,089	8	22	9	25	0,781	0,422	0,234
Slight improvement	14	39	18	50	0,343	26	72	12	33	0,001	0,004	0,151
Moderate improvement	11	31	13	36	0,617	2	6	14	39	0,001	0,006	0,808
Marked improvement	-	-	-	-	-	-	-	1	3	0,314	-	0,314
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Erythema												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	11	31	21	58	0,018	13	36	6	17	0,061	0,617	<0,001
Slight improvement	14	39	14	39	1,000	16	44	16	44	1,000	0,633	0,633
Moderate improvement	9	25	1	3	0,006	7	19	14	39	0,070	0,571	<0,001
Marked improvement	2	6	-	-	0,151	-	-	-	-	-	0,151	-
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Hydration												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-

Continue

Table 2: Evaluator physician's subjective criteria for clinical analysis before and after 90 days of using phytoestrogens-based product in volunteers with signs of photoaging. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

	Group A				Group B				Statistical significance for comparison between groups	
	D30		D90		Statistical significance Group A	D30		D90		Statistical significance Group B	D30	D90
	N.	%	N.	%		N	%	N	%			
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	-	-	-	-	-	-	-	-	-	-	-	-
Slight improvement	4	11	7	19	0,326	9	25	-	-	0,001	0,126	0,005
Moderate improvement	30	83	28	78	0,551	24	67	31	86	0,052	0,102	0,358
Marked improvement	2	6	1	3	0,555	3	8	5	14	0,453	0,643	0,088
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Vitality												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	1	3	1	3	1,000	2	6	-	-	0,151	0,555	0,314
Slight improvement	7	19	17	47	0,012	12	33	16	44	0,334	0,181	0,813
Moderate improvement	27	75	18	50	0,028	20	56	19	53	0,813	0,083	0,814
Marked improvement	1	3	-	-	0,314	2	6	1	3	0,555	0,555	0,314
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Oleosity												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	1	3	2	6	0,555	0,314	0,151
Unchanged	5	14	20	56	<0,001	2	6	22	61	<0,001	0,233	0,633
Slight improvement	14	39	15	42	0,810	20	56	11	31	0,032	0,157	0,326
Moderate improvement	17	47	1	3	<0,001	12	33	1	3	0,001	0,230	1,000
Marked improvement	-	-	-	-	-	1	3	-	-	0,314	0,314	-
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Smoothness												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	-	-	-	-	-	2	6	-	-	0,151	0,151	-
Slight improvement	10	28	8	22	0,586	10	28	-	-	0,001	1,000	0,003
Moderate improvement	23	64	28	78	0,195	23	64	32	89	0,013	1,000	0,206
Marked improvement	3	8	-	-	0,077	1	3	4	11	0,164	0,303	0,040
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-
Overall appearance												
Total worsening	-	-	-	-	-	-	-	-	-	-	-	-
Marked worsening	-	-	-	-	-	-	-	-	-	-	-	-
Moderate worsening	-	-	-	-	-	-	-	-	-	-	-	-
Slight worsening	-	-	-	-	-	-	-	-	-	-	-	-
Unchanged	1	3	-	-	0,314	2	6	-	-	0,151	0,555	-
Slight improvement	17	47	12	33	0,230	19	53	3	8	<0,001	0,637	0,009
Moderate improvement	18	50	24	67	0,151	15	42	33	92	<0,001	0,478	0,009
Marked improvement	-	-	-	-	-	-	-	-	-	-	-	-
Total improvement	-	-	-	-	-	-	-	-	-	-	-	-

Table 3: Cutaneous tolerability profile of phytoestrogens-based product in volunteers with photoaging signs after 90 days of use. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

	Group A					Group B					Statistical significance for comparison between groups	
	D30		D90		Statistical significance Group A	D30		D90		Statistical significance Group B	D30	D90
	N.	%	N.	%		N	%	N	%			
Erythema												
Excellent	36	100	36	100	1,000	34	94	35	97	0,555	0,151	0,314
Good	-	-	-	-	-	2	6	1	3	0,555	0,151	0,314
Moderate	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-
Dryness												
Excellent	36	100	36	100	1,000	34	94	34	94	1,000	0,151	0,151
Good	-	-	-	-	1,000	2	6	2	6	1,000	0,151	0,151
Moderate	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-
Desquamation												
Excellent	34	94	34	94	1,000	33	92	36	100	0,077	0,643	0,151
Good	2	6	2	6	1,000	3	8	-	-	0,077	0,643	0,151
Moderate	-	-	-	-	-	-	-	-	-	-	-	-
Severe	-	-	-	-	-	-	-	-	-	-	-	-

Table 4: Progression of ultrasonographic dermal density: before and after 90 days of using phytoestrogens-based product in volunteers with photoaging signs. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

	Group A					Group B					Statistical significance for comparison between groups	
	D30		D90		Statistical significance Group A	D30		D90		Statistical significance group B	D30	D90
	N.	%	N.	%		N	%	N	%			
Increased	24	66,7	19	52,8	0,225	20	60,6	24	72,7	0,292	0,601	0,079
Unchanged	12	33,3	17	47,2	0,225	13	39,4	9	27,3	0,292	0,601	0,079

DISCUSSION

Although the precise mechanism through which the skin ages is not fully understood, it is known that the reduction in the skin's immunomodulatory activity with age is one of its main causes – together with the reduction in the function and number of fibroblasts, and the destruction of important structures, especially collagen, elastin, and fibronectin. [13,14]

In addition to causing skin aging, the immunological alterations that occur with the aging process, also generate increased susceptibility to infections and cancer, since there are changes in immune activity. Among them is an alteration in the production of cytokines, which from a pro-inflammatory pattern (Th1), with the production of IL-1, becomes predominantly anti-inflammatory (Th2), with IL-10 as the dominant humoral response, which generates an exacerbated immunosuppression and reduction of the dermal and epidermal metabolism, and an acceleration of the aging process. [13,14]

In the findings of the in vitro phase of the study, even after the introduction of a substance that mimics chronological aging (LPS), it became evident that the phytoestrogens complex favored an increase in IL-1, so that baseline levels of that pro-inflammatory cytokine returned to those of the control, i.e. similar to those not influenced by the introduction of the aging simulator (Graph 3). Moreover, the complex also significantly reduced the

Table 5: Progression of dermal histology: before and after 90 days of using a phytoestrogens-based product in volunteers with photoaging signs. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

	Cutaneous ultrasound		Increased	Unchanged	Decreased
Collagen fibers	Group A	N	10	24	1
		%	27,80	66,70	2,80
	Group B	N	7	27	1
		%	19,40	75	2,80
	Statistical significance for comparison between groups		0,405	0,437	1,000
Elastic fibers	Group A	N	6	21	8
		%	16,70	58,30	22,20
	Group B	N	6	18	11
		%	16,70	50	30,60
	Statistical significance for comparison between groups		1,000	0,478	0,422
Mucin	Group A	N	-	22	13
		%	-	61,10	36,10
	Group B	N	1	22	11
		%	2,80	61,10	30,60
	Statistical significance for comparison between groups		0,314	1,000	0,617

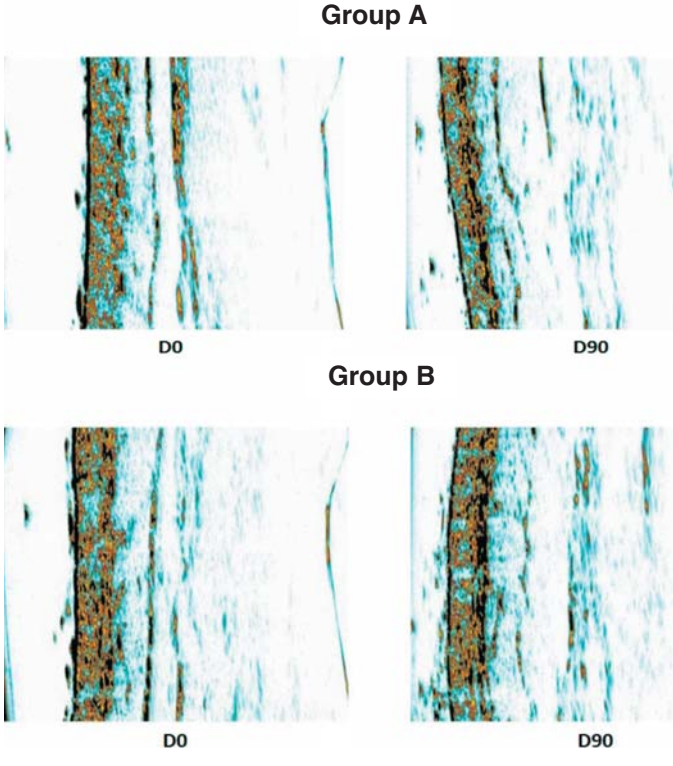


Figure 4: Evolution of ultrasonographic dermal density before and after 90 days of using phytoestrogens-based product in volunteers with photoaging signs. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

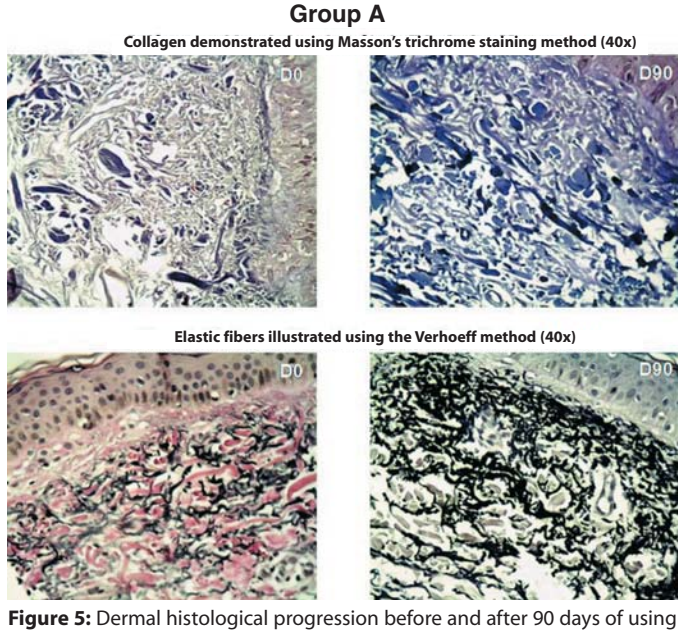
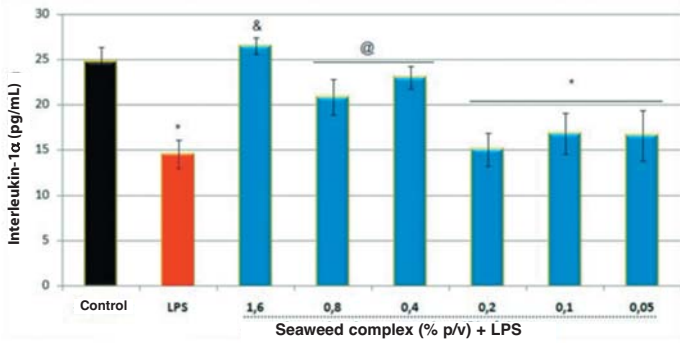


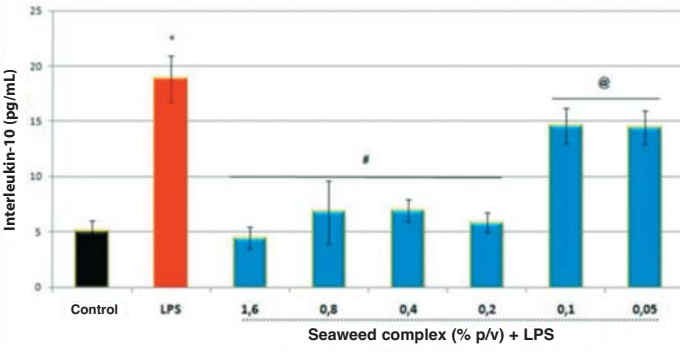
Figure 5: Dermal histological progression before and after 90 days of using phytoestrogens-based product in volunteers with photoaging signs. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

levels of anti-inflammatory cytokine IL-10 in the human keratinocytes cultures, keeping the 0.2, 0.4, 0.8, and 1.6% concentrations with values similar to those of the baseline control group (a reduction of approximately three times compared to LPS) (Graph 4). This demonstrates the complex's potential immunomodulatory stimulus, which promotes skin homeostasis – which is physiologically disturbed with aging.

The collagen fibers that provide a healthy structure and mechanical properties clearly decrease with the aging process. [8] The increase in procollagen (caused by the studied phytoestrogens) suggests a reconstitution of aged skin. In the histological analysis of the in vitro study with Sirius Red staining, an increase in collagen synthesis and better dermal re-densification, filling, and organization of the dermis was observed (Figure 3).



Graph 3: Phytoestrogen complex's effects on the synthesis of IL-1 in cultured human keratinocytes chronically stimulated with LPS



Graph 4: Phytoestrogen complex's effects on the synthesis of IL-10 in cultured human keratinocytes chronically stimulated with LPS

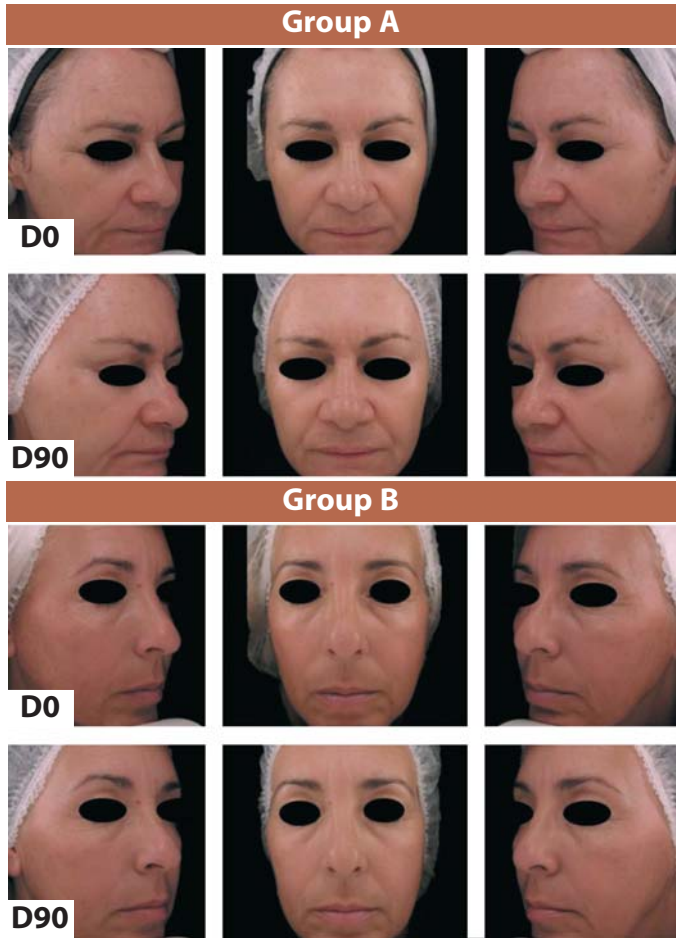


Figure 6: Clinical progression before and after 90 days of using phytoestrogens-based product in volunteers with photoaging signs. Group A) isolated use, Group B) used in combination with commercial cosmeceutical product

The complex has demonstrated the ability to significantly increase the relative expression of mRNA for procollagen in concentrations of 0.2, 0.1, and 0.05%; the highest concentration increased procollagen expression by 3.5 times (Graph 2).

The ability to increase the relative expression of mRNA for procollagen generates the production of more functional collagen "again" via an enzymatic reaction: the procollagen is cleaved in the skin by the procollagen enzyme peptidase, and becomes functional collagen in a directly proportional manner. 18 The data obtained in this clinical study demonstrate that the use of cosmeceuticals based on phytoestrogens complex can contribute considerably to preventing and reversing the signs of aging skin, acting directly and effectively in that enzymatic cycle.

In addition to exerting the capacity to contract and organize connective tissue, fibronectin promotes cell adhesion in wound healing and re-epithelialization, and is also primarily responsible for the integrity of the DEJ. [9-11] Fibronectin is one of the main markers of aging, when there is increased enzymatic degradation and a significant reduction in its amount and quality. [10,12]

The phytoestrogens complex was able to significantly increase the expression of mRNA for fibronectin, compared to the control group (Figure 1). An increase in the fluorescence intensity of the anti-fibronectin signal in the DEJ was obtained in the immunofluorescence evaluation, demonstrating the complex's positive effect in restoring the DEJ's integrity (Figure 1), which was altered during the aging process.

The expression of fibronectin (in the form of mRNA) was observed in the incubation of human keratinocytes with the phytoestrogens complex in the concentrations of 0.1%, 0.05%, 0.025%, and 0.012%; the last two concentrations promoted the best response (an increase of 5.5 and 7 times, approximately) (Graph 1).

From the clinical point of view, a favorable clinical response could be observed with the use of the product, either in isolation or combined with another cosmeceutical compound (Figure 6).

With the increase in life expectancy, the cutaneous aging process is becoming more evident, and is further aggravated by sun exposure, which damages various cell components by changing their molecular and morphological characteristics. This in turn stimulates the need for therapies that can prevent and minimize these undesirable effects. [19]

In this study, the favorable clinical response obtained with the use of phytoestrogens to treat the skin was proven not only in vitro but also in vivo, using clinical, laboratorial, and instrumental techniques.

CONCLUSION

From the results obtained, it is possible to conclude that the phytoestrogens complex can contribute considerably to the treatment of cutaneous photoaging, since it incorporates the essential factors for maintaining the quality of the skin. Its benefits are not restricted to the in vitro findings, but also extrapolated to the clinical domain and were supported by the complementary laboratory evaluations. The histologic findings obtained from the skin biopsies of study participants prove that the in vitro results of using the studied formulation are possible. It is important to observe the physio-temporal limitations typical of the human body's clinical responsiveness to any treatment. ●

REFERENCES

1. Makrantonaki E, Zouboulis CC. Molecular mechanisms of skin aging: state of the art. Ann N Y Acad Sci. 2007;1119:40-50.
2. Slominski A, Wortsman J. Neuroendocrinology of the Skin. Endocrine Rev 2000;21:457-87.
3. Trautinger F. Mechanisms of photodamage of the skin and its functional consequences for skin ageing. Clin Exp Dermatol. 2001;26:573-77.
4. Ma W, Wlaschek M, Tantcheva-Poor I, Schneider LA, Naderi L, Razi-Wolf Z, et al. Chronological ageing and photoageing of the fibroblasts and the dermal connective tissue. Clin Exp Dermatol. 2001;26:592-99.
5. Zouboulis ChC. Intrinsic skin aging. A critical appraisal of the role of hormones. Hautarzt. 2003;54(9):825-32.
6. Robert L, Fodil-Bourahla I, Bizbiz L, Robert AM. Effect of L-fucose and fucose-rich polysaccharides on elastin biosynthesis, in vivo and in vitro. Biomed Pharmacother. 2004;58:123-28.
7. Robert L, Fodil-Bourahla I, Bizbiz L, Robert AM. Effects of L-fucose and fucose-rich oligo and polysaccharides (FROP-s) on collagen biosynthesis by human skin fibroblasts. Modulation of the effect of retinol, ascorbate and alfa-tocopherol. Biomed Pharmacother. 2004;58:65-70.
8. Tzaphlidou, M. The role of collagen and elastin in aged skin: an image processing approach. Micron. 2004;35:173-77.
9. Wierzbicka-Patynowski I, Schwarzbauer JE. The ins and outs of fibronectin matrix assembly. J Cell Sci. 2003;116(16):3269-76.
10. Ruoslahti E. Proteoglycans in cell regulation, J Biol Chem. 1989;264(23):13369-72.
11. Rocquet C, Bonte F. Molecular aspects of skin ageing - recent data. Acta APA. 2002;11(3): 71-94.
12. Pieraggi MT, Julian M, Bouissou H, Stocker S, Grimaud JA. Dermal aging. Immunofluorescence study of collagens I and III and fibronectin. Ann Pathol. 1984;4(3):185-94.
13. Agius E, Lacy KE, Vukmanovic-Stejic M, Jagger AL, Papageorgiou AP, Hall S,et al. Decreased TNF-alpha synthesis by macrophages restricts cutaneous immunosurveillance by memory CD4+ T cells during aging. J Exp Med. 2009;206(9):1929-40
14. Assaf H, Adly MA, Hussein MR. Aging and Intrinsic Aging: Pathogenesis and Manifestations. In: Farage MA, Miller KW, Maibach HI. Textbook of Skin Aging. Berlin: Springer-Verlag; 2010. p. 130-132.
15. Fisher GJ, Talwar HS, Lin J, Voorhees JJ. Molecular mechanisms of photoaging in human skin in vivo and their prevention by all-trans retinoic acid. Photochem Photobiol. 1999;69(2):154-57.
16. Schieke SM. Photoaging and infrared radiation. Novel aspects of molecular mechanisms. Hautarzt. 2003;54(9):822-24.
17. Scharffetter-Kochanek K, Brenneisen P, Wenk J, Herrmann G, Ma W, Kuhr L, Meewes C, Wlaschek M. Photoaging of the skin from phenotype to mechanisms. Exp Gerontol. 2000;35(3):307-16.
18. Miller EJ, Gay S. The collagens: Na overview and update. Methods Enzymol. 1987;144:3-41.
19. Montagner S, Costa A. Bases biomoleculares do fotoenvelhecimento. An Bras Dermatol. 2009; 84(3): 263-69.