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REVIEW on PhD Thesis
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Research on "in vitro" processes modulation by certain natural compounds in the algorithm of skin tissue functionality

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Objectives and purpose of the work

In the area of modern cosmetics, the main objective is the development of therapeutic strategies to bring major benefits for the skin tissue status by targeted action of the active ingredients at gene or cellular receptor level, detaching it from the cleaning effect and apparent beauty characteristic so far for this type of products. In this context, this research aim was to highlight the dermo-epidermal mechanisms controlling the functionality of skin tissue homeostasis and the modulation of these processes by the specific plant origin compounds with pharmacological activity.

One of the main objectives of these studies focused on **defining the main cellular processes according to the therapeutic target**, as follows: at the epidermis level, the first layer of skin with barrier function against the external environmental aggression (keratinocytes) - inflammation, oxidative stress the initiation of apoptosis under UV radiation impact; at the dermis level, the region responsible for the appearance of wrinkles (fibroblasts) – the proliferative status, extracellular matrix protein synthesis, molecular expression of the type $\alpha 1\beta 1$ and $\alpha 2\beta 2$ integrins; vascular endothelium level, with a major role in skin homeostasis by providing oxygen, nutrients and hormones (human endothelial cells) - inflammation, angiogenesis.

Another objective was **to develop the therapeutic strategies** differentiated according to the previously defined specificity of the target mechanism, based on the

exploitation of some natural sources, in order to obtain classes of bioactive compounds with proven efficacy. Protection mechanisms against external environmental aggression and antiageing skin reconstruction were modulated using individual or combination of active ingredients.

The experimental models designed and implemented to test these active principles demonstrated the strictly directed effectiveness of the bioactive substances according to the induced specificity on the cellular processes dynamic monitored.

The experimental methods used in defining the mechanisms of action of the bioactive compounds are modern multiparameter cellular investigative techniques (flow cytometry analysis oriented both to staining and fluorescent analyzing the cell parameters and to detect soluble proteins from the culture medium using a technique based on fluorescent particles coupled with antibodies - "beads-based assay"; analysis of the cell morphology by inverted optical microscopy; spectrophotometry). The experimental methods used for defining the mechanisms of action of the bioactive compounds are modern multiparameter cellular investigative techniques (flow cytometry - pharma geared towards marking and analysis of fluorescent cell parameters and to detect soluble proteins from the culture medium by technique based on fluorescent particles coupled antibody - "beads-based assay"; analysis of the cell morphology by inverted optical microscopy, spectrophotometry). They bring together complementary methodologies and correlative characterization of the specific cellular processes, complex simultaneous analysis of the main parameters defining skin functionality, creating requisites for the correct definition of the therapeutic targets for each compound and improve the efficacy of the future dermatocosmetics.

State of the art - cellular mechanisms involved in skin functionality

The skin is one of the largest organs, with an area of approximately $2m^2$ and weighing 16% of the body weight. Its functions are varied: barrier against microorganisms aggression, prevents dehydration, sensory organ, thermoregulation. The epidermis is the surface epithelial layer, the dermis is related tissue below the epidermis and hypodermis is the subcutaneous connective tissue (Young, 2000; Fawcett, 2002).

In the aging process, there are a number of complex molecular and cellular events and differentiated according to the external environment aggression (photo-aging) and the intrinsic specificity of the body (chronological aging). Cell proliferation and its correlation with various metabolic pathways (intracellular calcium, protein kinase C) is a relevant parameter for the studies "anti-aging" at the skin level (Soroka et al., 2008), being known to decrease the rate of multiplication and turn-over of the fibroblast cell associated with aging.

The dermis is composed mostly of collagen and elastin fibers. Thick collagen fibers support the skin. The elastin fibers are very flexible and gives strength and adaptability to the conformational changes of the skin. The dermis consists mostly of connective tissue, also known as extracellular matrix (ECM) secreted by fibroblasts.

ECM consists of three classes of biomolecules, with a wide variety of shapes and sizes: structural proteins: collagen and elastin, specialized proteins: fibrillin, fibronectin and laminin, proteoglycans. Considering that the collagen type I and III are dominant in the skin, using agents and treatments to stimulate the synthesis of collagen typeswould be of a great interest. It also might be used to inhibit collagen degradation as a single method or in conjunction with stimulation of the synthesis. Lots of research has focused on finding effective topical inhibitors of the metalloproteinases (particularly: collagenase), while the modulators at the molecular level and even gene (eg activation/ inhibition of expression of TGF-β) is the target of the latest innovative anti-aging dermatocosmetic formulations. The profound effect of TGF-β in the extracellular matrix homeostasis is due to its ability to alter the balance of activation / inhibition of the matrix metalloproteinase (MMP) at the gene expression level. TGF-β1 is the major isoform, predominant in the tissues and intercellular fluid, involved in the wound healing process, with a role in stimulating the synthesis of collagen, matrix turn-over modulation by inhibitory effects on MMP and by inducing the differentiation of myofibroblasts, a cell type occurring in the wound contraction process, the synthesis of granulation tissue constituents, including formation of the collagen and fibronectin in the new dermal composite (Rhein, 2010).

Another important aspect is the imperfect healing after the sun exposure or minor trauma due to the deposits consisting of disorganized fibers of collagen and elastin. The therapeutic intervention in this case should take place in two ways: **enzymatic degradation** of these clusters under the **activated metalloproteinases action** and **the stimulation of the protein synthesis** to regenerate the structural support of the extracellular matrix. In addition to this process, was recently discovered a convergent mechanism at α1β1 and α2β1 integrins involved in creating links cell - extracellular matrix protein, enhancing their expression at dermal fibroblast level supporting skin firmness restoration.

The role of estrogen in the skin is recognized, acting through their cellular receptors on the proliferation and collagen synthesis (Eui Dong Son et al., 2005).

The skin may be an interface between the body and the environment, acting as a barrier against the damaging effects of the exogenous chemical and physical agents, including UV radiation. (Carini et al., 2000). The photo-oxidative stress originating from the reactive oxygen species formed in sun-exposed skin is a key mechanism, with effects at the level of degradation of the extracellular matrix and wrinkle formation, as well as in cellular photo-mutagenesis involved in skin carcinogenesis. Photo-aging, caused by UV radiation generates free radicals at the cellular level, has physiological features distinct from the chronological age. Given the above, one of the target actions of an dermatocosmetic ingredient should be the oxidative stress and triggered cellular chain reactions reduction: the apoptosis induction, pro-inflammatory cytokines secretion, aberrant vascularization promoted at the molecular level by the VEGF (Wondrak, 2007).

Traditional approaches on the skin aging process appear incomplete, mainly due to the significant changes in the dermo-epidermal junction, in the assessment of the key

proteins. The integrins, part of this class of proteins, are also cell surface receptors responsible for the transfer of information between the interior and exterior of the cell. The integrins are involved in various cellular functions, from the growth and development, to the immune response and wound healing. The α1β1 integrin mediates feed-back control of the collagen synthesis, in order to create links cell- collagen or cell-laminina1 type in the extracellular matrix and the integrin α2β1 mediates stimulation of the type I collagenase (MMP1) involved in fibrillogenesis (fibrils organization of the collagen) and binds type I collagen. Balance between the α1β1 and α2β1 integrin is important for maintaining the balance between degradation and synthesis of collagen. (Riikonen et al., 1995; Zhang et al., 2006; Fujimura et al., 2007).

Endothelial cells in the dermis are involved in wound healing, inflammation, tumor angiogenesis, and are predominantly micro-vascular origin, having the different provenience and functionality versus endothelial cells from large vessels, used for the *in vitro* vascular research. The increased endothelial cell turnover in the skin is essential not only in the normal development, but in wound healing, hair follicle cycle, tumor cell metastasis, and various stages of cutaneous pathology. There are several aspects of the physiology of dermal vasculature and its involvement in skin tissue homeostasis. Among these are: *vascular inflammation*, characterized by the adhesion between lymphocytes and endothelium triggered by the expression of adhesion molecules - markers of inflammation and pro-inflammatory cytokine secretion; the *involvement of certain cytokines*, essential in the endothelial activation, injuries healing caused by UV radiation or skin wound, the *phenomenon of angiogenesis* and cell proliferation.

State of the art - natural compounds involved in modulating cellular dermo-epidermal processes

Natural sources contain a wide variety of organic compounds which are usually classified as primary and secondary metabolites of different organisms. Primary metabolites are compounds that have a key role in photosynthesis, respiration, growth and development. This classification could include phytosterols, lipids, acyl nucleotides, amino acids, organic acids.

The other compounds are usually in very small amounts, but which, surprisingly, in some species accumulate in high concentrations, are secondary metabolites. They have different structures (some distributed in a limited number of plant species in the world), ignored for a long time, but lately becoming of great interest, having demonstrated key rols in protecting plants against microbial infections, in attracting pollinators, in UV protection, or the nitrogen fixation. These metabolites are also interesting because of the possible use as colorants, fiber, bonding agents, oils, waxes, and fragrance agents representing new natural sources for new medicines, antibiotics, insecticides and herbicides.

Many biologically active substances with known effects on human physiology have been identified from data provided by traditional medicine and recently used both for the therapeutic and cosmetic purposes. Chemistry of the natural compounds forms not only the scientific basis of the use of medicinal plants, but also plays an important role in the discovery of active cosmetic ingredients, pharmaceuticals and nutraceuticals.

State of the art - maintaining good function of the cutaneous tissue as a result of the active treatment at the skin level

The status of the skin is generally defined as a combination of the physiological characteristics, color and texture related with the sebum content, the moisture level and the skin epidermis pH. The presence of an appropriate amount of water in the *stratum corneum* is important for the smooth and soft appearance of the skin. The sebum secreted by the sebaceous glands along with other epidermal lipids helps maintaining the moisture by creating a protective layer on the skin surface that reduces the fluid loss through the epidermis. In addition, the lipids together with the amino acids contributing to the maintenance of the skin acidity and the low pH of the skin protects agains microorganisms aggression.

The dermatocosmetic field main objective focusses on finding solutions bordering the therapy, to restore physiological balance of the key parameters, based on specific investigations in the dermis and epidermis.

Advanced research aims developing products to **improve hydration of the *stratum corneum* and the hydro-lipidic barrier and integrity based on the biology of the dehydrated *stratum corneum***. This led to highlight the cellular and molecular mechanisms which substantiated the development of the dermatocosmetic ingredients with specific biological target.

Also, the cellular adhesion presents new insights on restoring skin firmness (Moreau, 2006). Skin firmness is at the molecular level, the consequence of the cell adhesion and integrin expression of the fibroblasts individual products.

Cell interaction with their environment is mediated by specific receptors on their surface. The extracellular matrix of the skin, a family of membrane receptors called integrins, play an important role in the intercellular and cell-protein adhesion and in the extracellular matrix organization and assembly on which depends skin firmness.

We have studied the adhesion capacities and the cell membrane integrins expression in the presence or absence of the UV radiation exposure of the cells.

Inflammation induction is often associated with the immune dysfunction, this condition difficult to treat being a self-induced phenomenon going through a cycle of amplification. In cosmetic applications, inflammation may have multiple causes, such as aging, UV radiation, pollution. Long term exposure to these factors induce a phenomenon of skin irritation manifested by slightly activating but progressive, of the proinflammatory cytokines, keratinocytes turn-over acceleration, affecting their function and homeostasis, disturbing the structure of the *stratum corneum*, which increases the risk of penetration of the external agents aggressors.

Introduction

It is well known the negative impact of the products which use aggressive marketing strategy without relying on eloquent biological tests, inducing in the consumer population the initiation of the long-term dermatological disorders (allergic reactions or sensitization to certain substances, dermatitis, etc.), or not deliver the promised benefits. Involving research on this issue is very important, that bringing the elucidation of biological processes at the dermo-epidermal tissue and demonstrating the potential toxicity and / or efficacy of certain active compounds according to a custom physiological mechanism, as well as highlighting some interactions between the different active ingredients that may potentiate or inhibit the target therapeutic effect.

In the context of an explosion of products on the international dermatocosmetics market and the diversification of the declared effects on the one hand, as well as the existence of consumers becoming more demanding and educated, this study aims to fill an area of interest in the scientific research, dermatocosmetic manufacturers and the general public, by bringing out the cellular and molecular mechanisms involved in skin tissue functionality and highlighting the therapeutic potential of the active ingredients of plant origin.

The interdependence of the cellular and molecular processes and the specific regulation of the signaling pathways to initiate physiological control mechanisms constitute an algorithm that defines the functionality of a particular type of tissue and is then integrated in the global response of the body on the tolerance and efficacy of a treatment.

In this context, we propose a full screening in the main cell types that form or support the skin tissue activity: keratinocytes, fibroblasts and vascular endothelial cells, the modulation of their functionality using some isolated and purified biologically active compounds from the species belonging to the indigenous flora with phytotherapeutic potential.

Cellular functionality would be defined by investigating and correlating of the complementary parameters specific to each type of pursued process, eg:

- **concerning the keratinocyte**, the main line of defense of the body against the external environment aggression, we selected an experimental model of UV iradiation in which we highlight the impact of the treatment with certain bioactive compounds on the apoptosis, cell cycle sequencing, the main pro-inflammatory cytokines (IL6 and IL8) and pro-irritative (IL 1α), cellular oxidative stress (expressed as hydrogen peroxide and superoxide anion released intracellular) and pro-angiogenic factor VEGF;
- **concerning the fibroblast**, with important structural and functional role especially in aging, we selected explorative studies in normal conditions and estrogen depletion highlighting: the cellular proliferative status correlated with modulation of intracellular calcium influx and regulatory molecules of collagen formation (TGFβ) and membranary expressed molecules playing a role in the extracellular matrix-cell adhesion (integrins α1β1 and α2β1);

- **concerning the vascular endotelium,** involved in inflammatory processes associated with the skin diseases (eg acne, edema of different etiologies, etc) have been investigated the representative parameters under this pro-inflammatory cytokines (IL6 and IL8), endothelial adhesion molecules - monocyte (ICAM and VCAM) and factor VEGF (vascular endothelial growth factor) as an indicator of vascular leak.

The natural compounds, potential modulators of these processes were selected based on the bioactive components contained and their directly impact on the target cellular mechanisms. Thus, we have been tested the following standardized extracts and purified substances:

- **Dermo-ET** - effect at the fibroblast and keratinocyte level
- **Dermo-P, Dermo-Egr, Dermo-L** - photoprotection effect at the keratinocyte level
- **Dermo-Dis, Dermo-Ska, Dermo-Sks** - effect at the fibroblast and keratinocyte level
- **Dermo-Es, Dermo-HdC, Dermo-Dis -** effect on vascular endothelial level.

Materials and Methods

Cell Culture

Demonstration of the biological effects of plant extracts was performed at the target cell, on the standardized cell lines relevant to the studied mechanism:

- **Effect of restructuring dermal (cell turn-over and skin tissue firmness restoration)** was tested on human dermal fibroblasts (HS 27), the main cell type responsible for both the synthesis of the structural proteins of the extracellular matrix, under strict signaling of the TGFβ factor and the integrins expression - adhesion molecules in the cell - matrix protein adhesion.
- **Anti-inflammatory effect at the vascular endothelium** assessed by using the specific line HUVEC (human umbilical vein endothelial cells) due to its relevance in simulating the inflammatory process at the level of blood vessel.
- **Photoprotection effect** has been shown in the human keratinocyte line HaCaT, this cell type forming the epidermis, the first layer of the skin tissue with a barrier function against the harmful effect of the UV radiation.

Methods of investigation

- Cytotoxicity was measured by determining the metabolic activity (MTS technique) and the release of lactate dehydrogenase (LDH) into the extracellular medium in response to a toxic stimulus (BARLTROP et al., 1991 RISS et al., 1992) (Fotakis TIMBRELL 2006).
- Determining the sequence of the cell cycle by flow cytometry (http://www.biology.arizona.edu/cell_bio/tutorials/cell_cycle/cells2.html)

- Highlighting the proliferating generation succession by fluorescent marking with the CFSE (carboxy-fluorescein-succinimidyl ester) (LYONS and DOHERTY 2004 LYONS 2000)
- Assessment of the apoptotic status by double fluorescence labeling and flow cytometry (MARTIN, et al., 1995).
- Flow-cytometric detection technique of the soluble analytes „BEADS-BASED ASSAY" (COOK, 2001).
- Demonstration of the over-expression of the adhesion molecules ICAM, VCAM, and glyco-protein chains α1, α2, and β1 of the integrins by fluorescent antibody labeling
- Determination of the hydrogen peroxide and superoxide anion by intracellular flow cytometry (CARINI et al., 2000; ROBINSON et al., 2009).
- Determination of the intracellular calcium by flow cytometry (fluorescenct marking FLUO 4) (VARANI et al, 1990; MONACO et al, 2009)

Results and Discussion

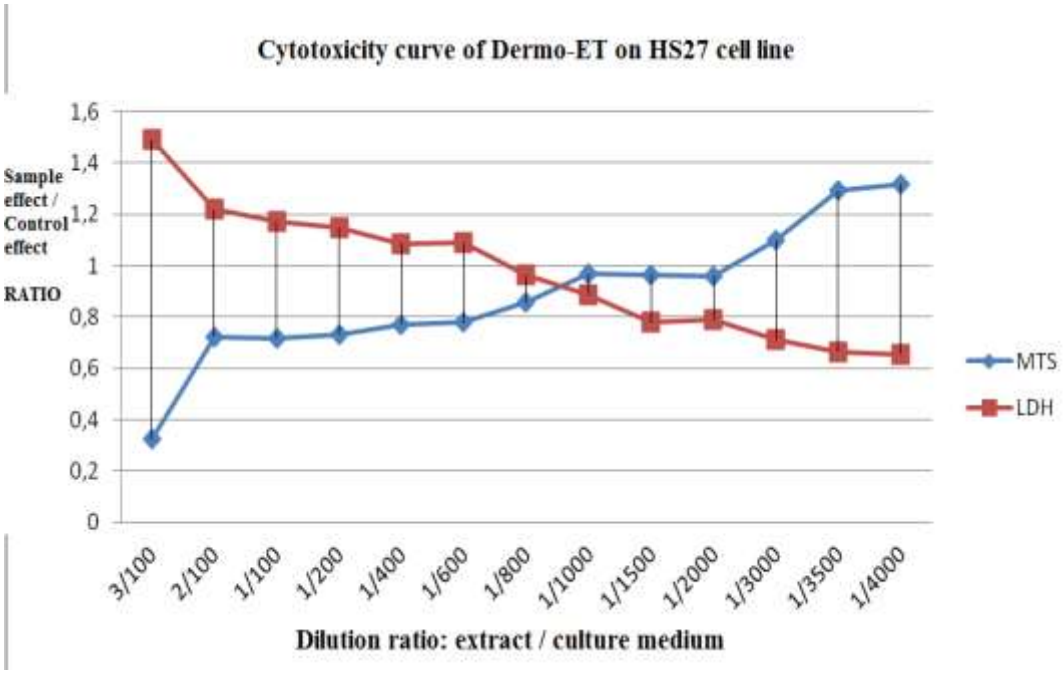
Phytocompounds' cytotoxicity depending on the type of the target cell

Cytotoxic to human dermal fibroblast (HS 27 standardized cell line)

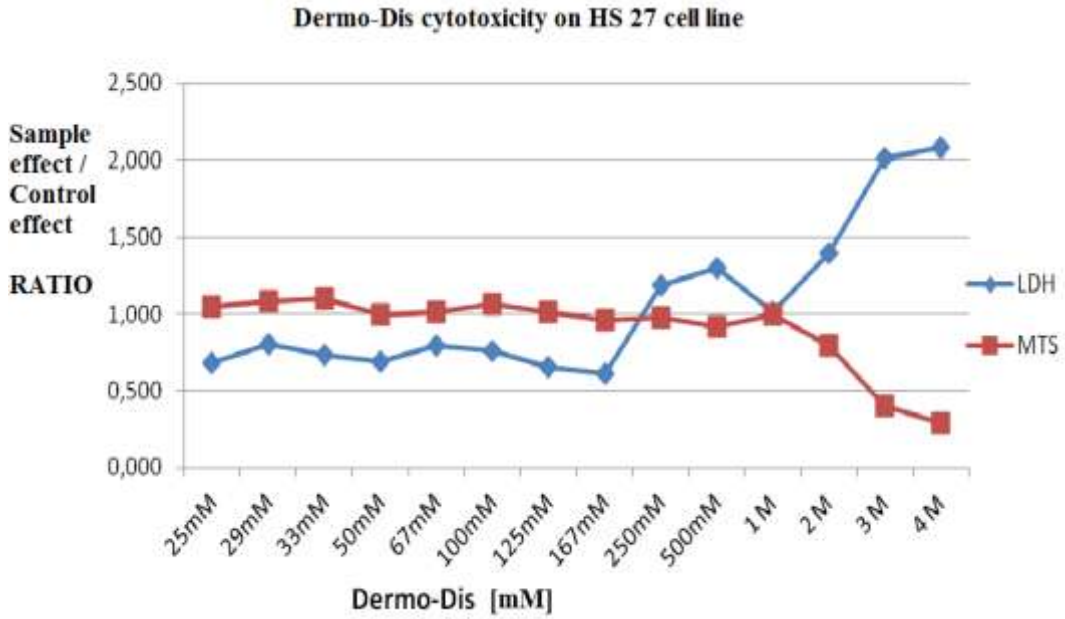
After the treatment with different concentrations of the extract for a period of 24 hours, the standardized HS27 line of normal fibroblast cells adhered for 24 hours, we measured the absorbances corresponding to the cellular metabolic activity (MTS) and to the accumulation in the culture medium of the stable cytosolic enzyme LDH, as a result of the toxic action compounds. The results are presented for each bioactive compound.

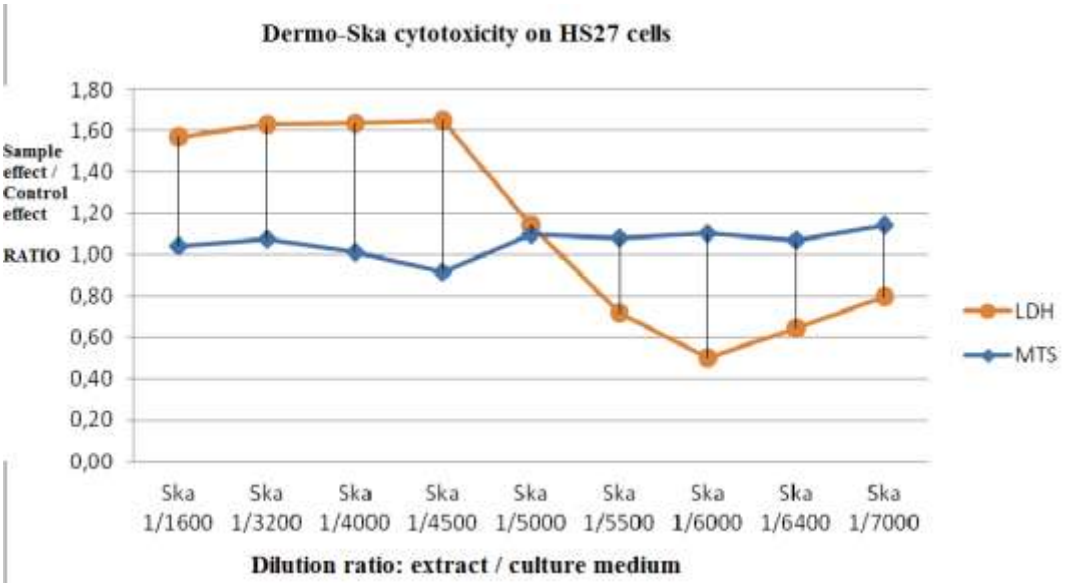
a) Biocomplex Dermo ET

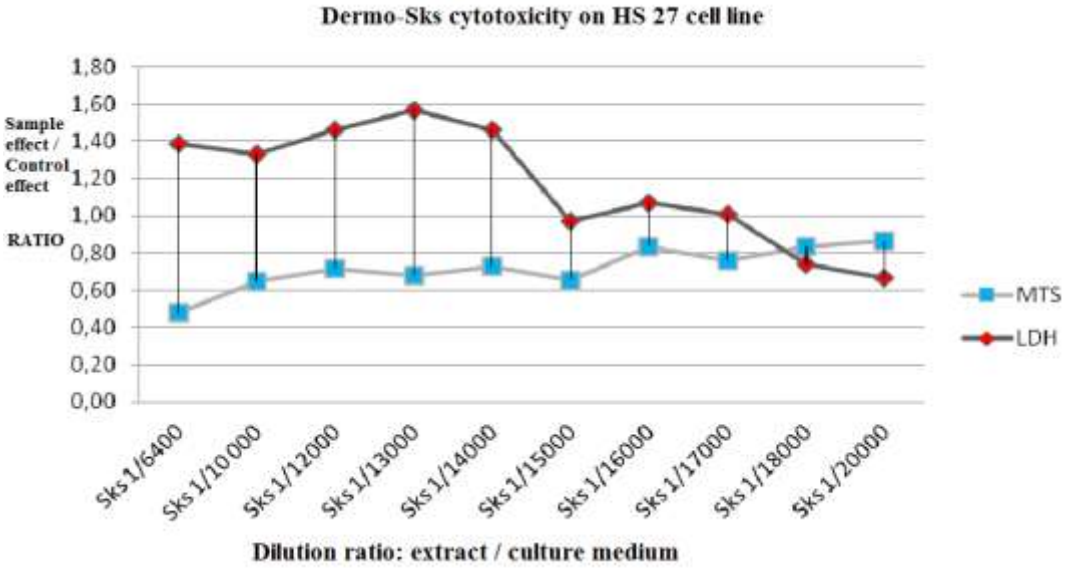
We consider optimal working dilutions on the specific cell line as dilutions greater than or equal to 1/800 Dermo-ET (this value represents the inflection point at which metabolic activity is increased and decreased secretion of lactate dehydrogenase, an indicator of cell toxicity).



b) Biocomplexes Dermo-Dis, Dermo SK-a, Dermo SK-s







Dermo Dis has the cytotoxicity dose limit of 170mM, efficacy trials could be configured from this concentration, in descending order.

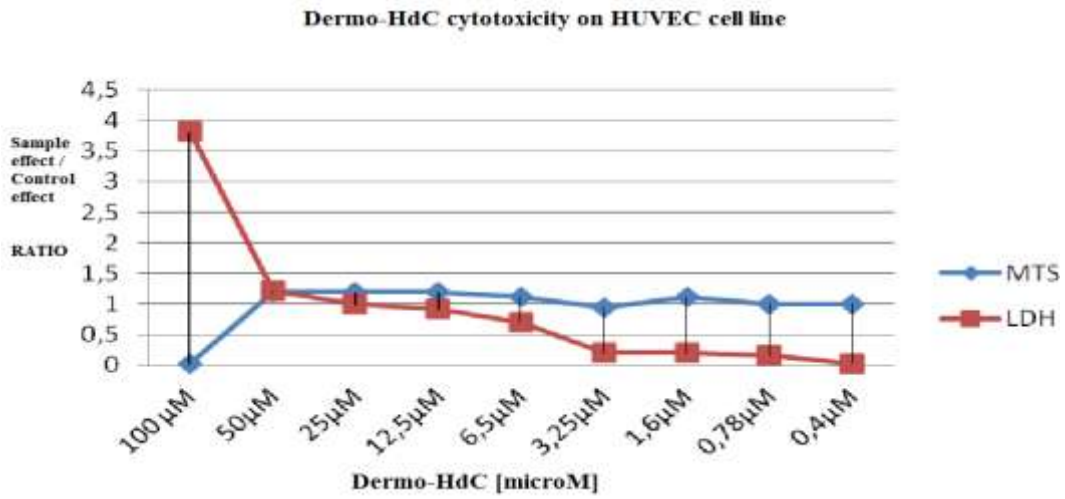
We consider the optimal working dilutions over 1/5000 Dermo-Ska for testing specific activity of the compound on normal human fibroblast line - HS27.

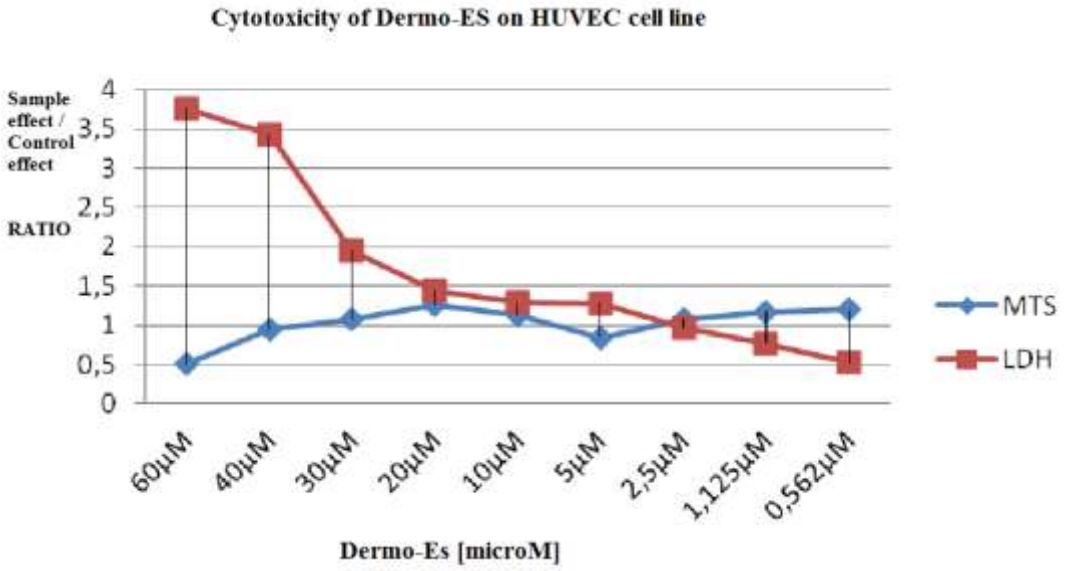
Dermo-SKs extract is more toxic than its counterpart containing hydrolyzed saponin (Dermo-Ska) and can be tested for specific activity at normal human fibroblasts to **higher dilution 1/18000**.

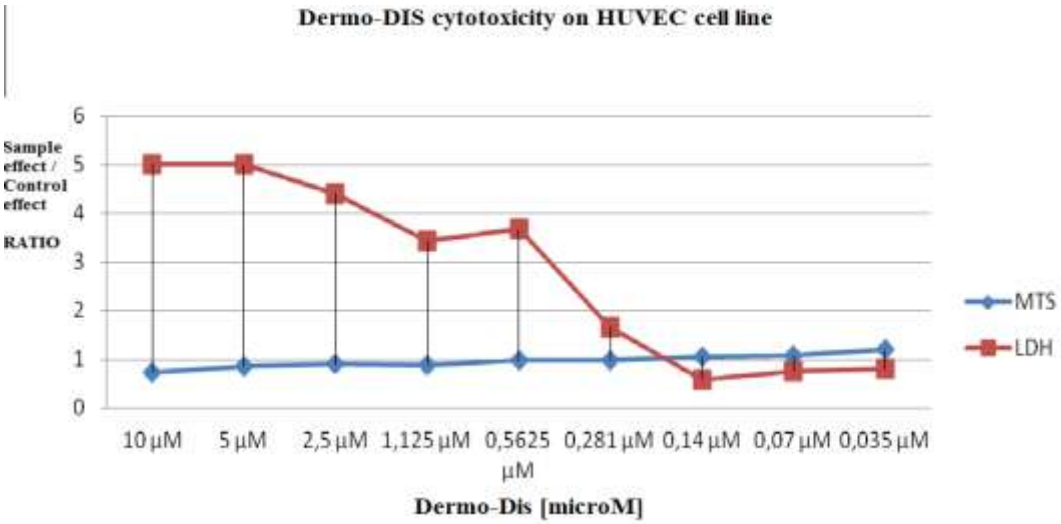
Cytotoxicity at the vascular endothelium level (HUVEC line)

After the treatment with different concentrations of the extract for a period of 24 hours, the HUVEC endothelial cell type vasculitis, adhered to 24 hours, we measured absorbances corresponding to the cellular metabolic activity (MTS) and accumulation in the culture medium of the stable cytosolic enzyme LDH following the action of the toxic compounds.

a) Biocomplexes DERMO-Hdc, DERMO-Es and DERMO-Dis







Dermo-HdC cytotoxic limit is to 50µM, the specific activity of the compound at the vascular endothelium may be assessed bellow that concentration.

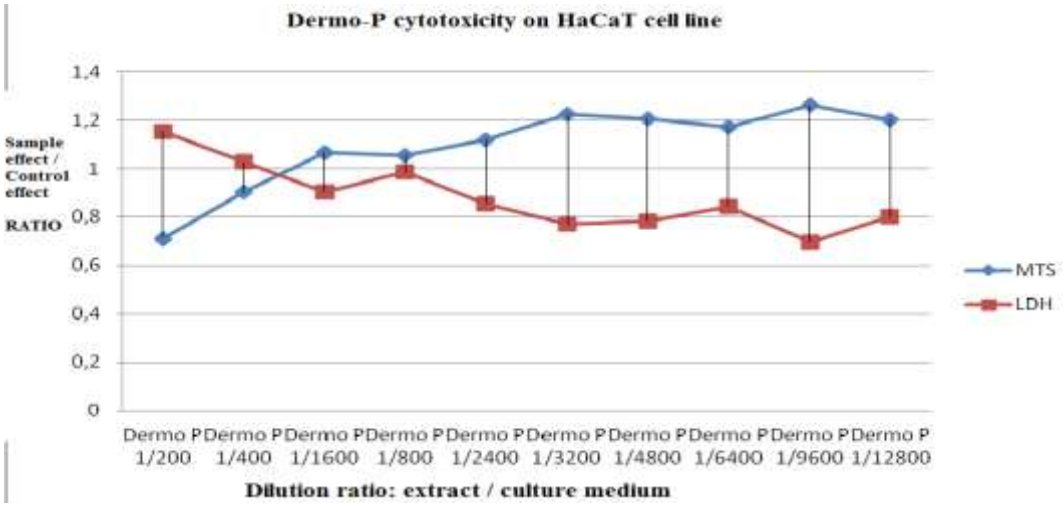
Cytotoxic threshold at the vascular endothelium level is **2,5 µM for the Dermo-Es.**

Specific activity tests for the Dermo-Dis on the HUVEC endothelial cell line could be run from a dose of 0.14 µM (140 nM).

Cytotoxicity on the normal human keratinocytes level (HaCaT)

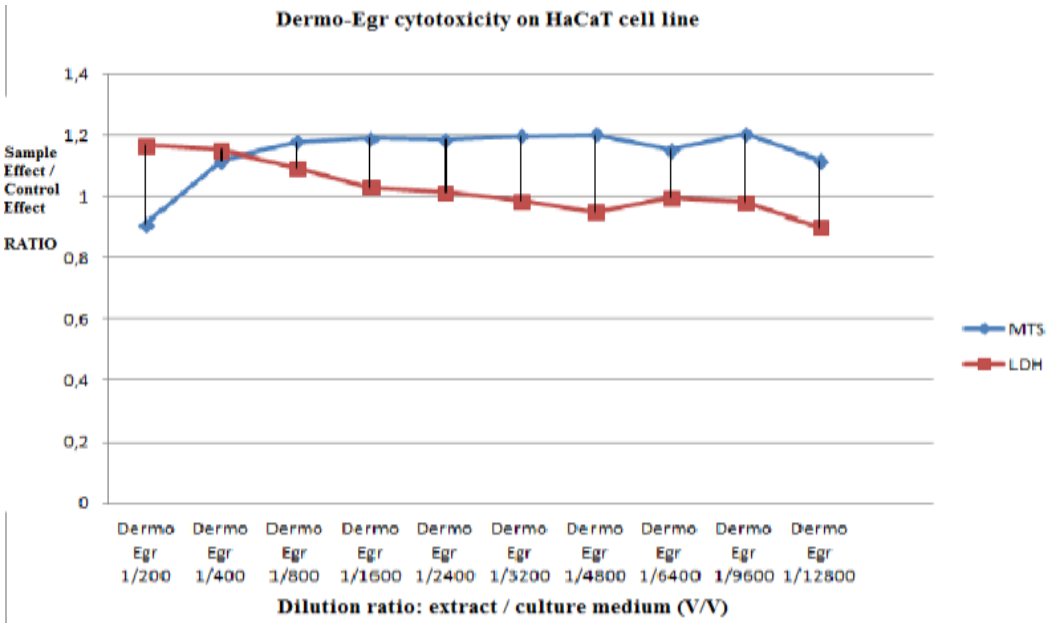
After the treatment with different concentrations of the extract for a period of 24 hours, normal human keratinocytes line standardized HaCaT, adhered for 24 hours, we measured the absorbance corresponding to the cellular metabolic activity (MTS) and accumulation in the culture medium of the stable cytosolic enzyme LDH as a result of the action of the toxic compounds.

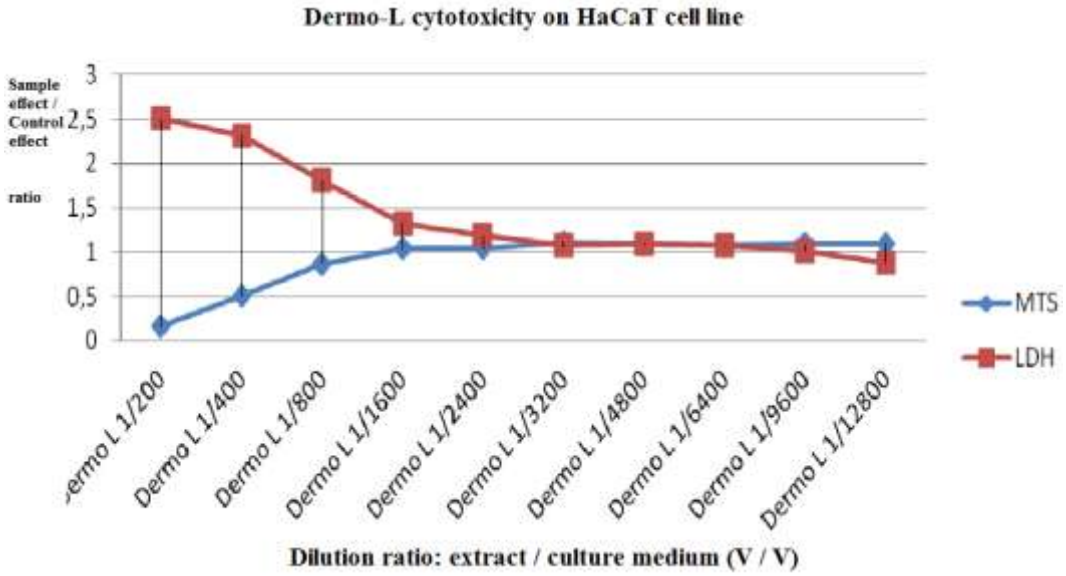
a) Biocomplex Dermo P



According to the cytotoxicity curve, the specific activity at normal human keratinocytes can be evaluated at dilutions greater than, or equal to **1/500 for the Dermo-P**.

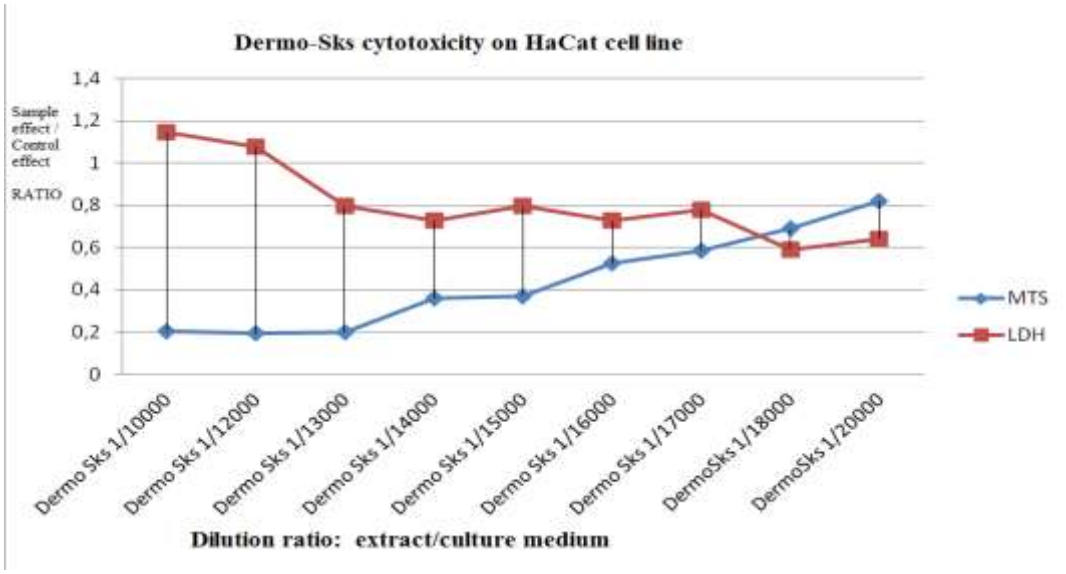
b) Biocomplexes Dermo Egr and Dermo L

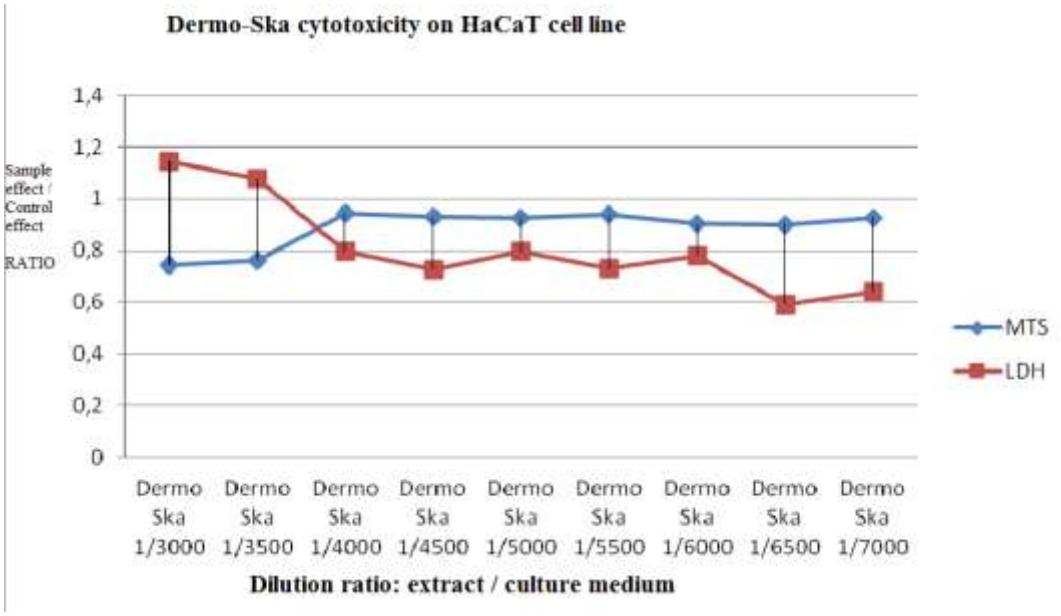




The citotoxicity curve for the **Dermo-Egr** revealed that the extract is non-cytotoxic to human keratinocytes at normal dilution of **1/500** or higher. The cytotoxic limit on the normal humankeratinocytes is **1/2000** for the **Dermo-L** extract.

c) Biocomplexes Dermo SK-a, Dermo SK-s

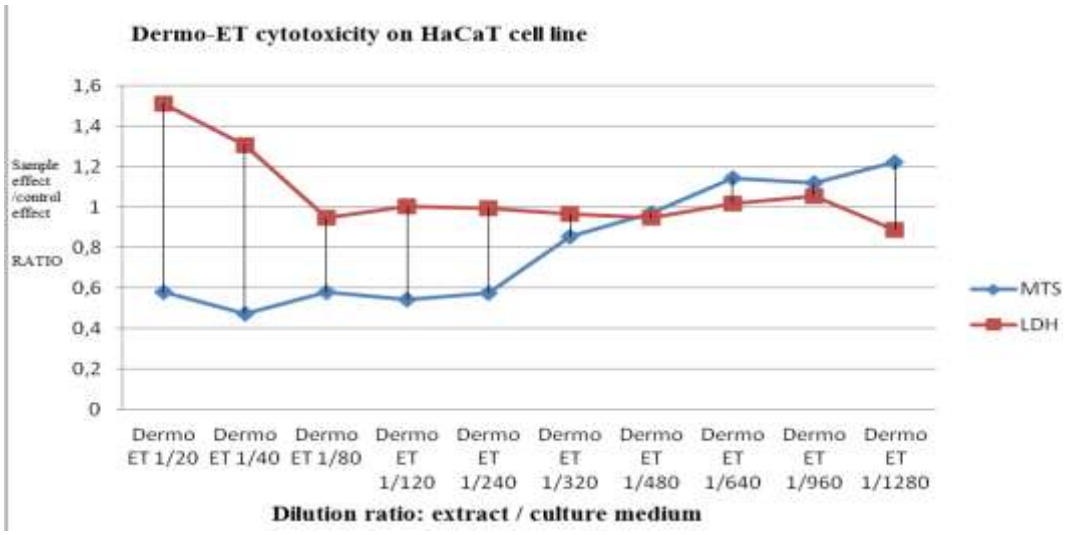




According to the cytotoxicity curves, the specific activity at the keratinocyte level can be evaluated for dilutions greater than, or equal to **1/18 000** for the **Dermo-Sks** extract. The cytotoxicity limit for the **Dermo-Ska** is **1/4000** at the normal human keratinocytes level.

d) Biocomplex Dermo ET

At the level of the normal human keratinocytes, the threshold of cytotoxicity is 1/480 for the Dermo-ET extract.



The compounds activity on the cellular proliferative status of the normal dermal fibroblasts by normal cultivating conditions as well as estrogen depletion; correlations with the intracellular calcium modulation

The effect has been shown in cultured human dermal fibroblasts (selective cell line for the followed process) by complementary data at the DNA synthesis level and the sequence in the division of proliferative generations. Knowing the role of the calcium in the activation of the important metabolic pathways and processes regulating cell division, some experiments included the determination of the intracellular calcium, in order to reveal possible mechanisms of action of the active principles studied.

The cellular proliferative status was analyzed by complementary techniques of analysis, namely: cell cycle sequencing and succession of the proliferative generations (flow cytometry with propidium iodide labeling and CFSE respectively - carboxy fluorescein diacetate succinic imidil ester).

The results were estimated as Proliferation Index, the sum of the percentages of cells in S and G2/ M phases of multiplication, calculated with a specific analysis software (FACS Express V3 DNA cell cycle and proliferation mode).

The experimental data (five successive experiments) demonstrate that both cell proliferation and cell cycle sequencing are adjusted to the estrogen receptor, the proliferative capacity decreasing under hormone depletion and amplifying with the action of β-estradiol, the synthetic equivalent.

Based on these observations we tested the effect of compounds at 48h. Action on two test series with/ without estrogen, compared with the positive control 17-β-estradiol for estrogenic action.

Dermo ET contains the active ingredients daidzein, genistein, formononetine and biochanine A, and its action has been evaluated compared with the activity of these components and their appropriate doses of the combination in the extract.

The tables below show the action of the compounds on the standardized human normal fibroblast cell lines (HS 27) compared with the positive control (β estradiol 1nM).

Table 1. Dermo-ET impact on the proliferative capacity of the dermal fibroblasts.

	medium containing normal estrogen levels				medium without estrogen			
	% phase S+G2/M	% variation	Prolife-ration index	% variation	% phase S+G2/M	% variation	Prolife-ration index	% variation
Control	23,5		3,5		18,5		2,4	
Solvent control	27,7		3,0		19,5		2,6	
Dermo ET 1/1000	28,9	23,0	4,2	19,4	25,7	39,1	4,3	79,2
Dermo ET 1/2000	25,3	7,8	4,2	18,6	25,1	35,6	3,9	62,5
Formononetin equivalent Dermo ET1/2000	49,7	111,5	2,8	-19,4	44,2	138,9	3,5	45,0
Formononetin equivalent Dermo ET 1/1000	48,4	106,0	3,2	-8,0	47,6	157,3	6,0	149,2
Biochanine A equivalent Dermo ET 1/2000	51,3	118,3	2,4	-32,0	48,7	163,2	3,8	56,3
Biochanine A equivalent Dermo ET 1/1000	47,2	100,9	3,9	10,6	47,3	155,7	6,0	150,8
Genistein equivalent Dermo ET 1/2000	29,4	25,1	3,8	9,1	30,0	62,2	4,0	67,9
Genistein equivalent Dermo ET 1/1000	17,4	-25,9	3,3	-5,4	23,1	25,0	3,8	58,3
Daidzeine equivalent Dermo ET 1/2000	46,1	96,2	2,6	-24,6	51,6	178,9	4,1	71,7
Daidzeine equivalent Dermo ET 1/1000	27,8	18,3	3,5	-0,6	44,5	140,5	4,1	71,7

(Daidzeina+ Genisteina+ Biochanine A+ Formononetin) equivalent Dermo ET 1/1000	56,1	138,7	5,4	53,4	50,6	173,5	3,5	45,8
(Daidzeine+Genisteine+Biochanine A+ Formononetin) equivalent Dermo ET 1/2000	54,0	129,8	5,1	45,1	48,8	163,8	3,6	49,6
17 β estradiol 1nM	53,6	128,1	3,48	-0,6	54,6	195,1	4,11	71,3

The Dermo-ET product stimulates growth rate of the fibroblast cell proliferation in normal cell development as well as in the estrogen-depleted conditions, similar to the synthetic estrogens, eg. β-estradiol. Estrogen-mimetic effect is demonstrated by each component of the isoflavones mixture, particularly daidzein, formononetin and biochanine A in the rate of DNA synthesis and initiation of mitosis in the division cell cycle.

The transmembrane transport activity was examined by the action of the ionophore A 23187 which opens calcium channels and ensures an influx of ions from the outside to the inside of the cell, in the case of high concentrations in the extracellular medium, or a decrease in the intracellular calcium concentration where the external environment of the cell is low in calcium ions compared to the intracytoplasmic medium.

Table 2. Modulation of the intracellular calcium (expressed as median channel fluorescent emission FITC-A) under the effect of Dermo-ET in culture media containing different estrogen levels

	medium without estrogen		medium containing normal estrogen levels	
	FITC-A without ionophore	**FITC-A with ionophore**	**FITC-A without ionophore**	**FITC -A with ionophore**
Cellular control	112	187	285	392
Solvent control	113	181	278	397
DERMO ET 1/1000	222	318	298	481
DERMO ET 1/2000	140	219	303	421
Genistein + Daidzein + Biochanine A + Formononetin equivalent DERMO ET 1/1000	170	289	258	302
Genistein+Daidzein + Biochanine A + Formononetin equivalent DERMO ET 1/2000	132	254	347	403
17 β estradiol	306	455	180	381

The fibroblast basal intracellular calcium concentration is lower in the extracellular medium, the experimental ionophore effect being to create an influx of calcium from outside to inside. Experiments performed at 48h showed a massive increase in intracellular calcium induced by **DERMO ET 1/1000**, that accelerates the cell proliferation of the tested human fibroblasts. It proved that the Dermo-ET complex action, estrogen-mimetic, with improved proliferative capacity of the dermal fibroblasts using calcium as secondary messenger for triggering and support this process. Ionophore A23187 enhances intracellular calcium accumulation by activating the flow of the extracellular environment by completing mobilization of this ion from intracellular compartments and / or influx induced by Dermo-ET (A00688 Patent Application, 2012).

Tests on Dermo-Ska and Dermo-Dis extracts: We analyzed the cellular proliferative status influenced by **Dermo-Dis** and **Dermo-Ska** through complementary analytical techniques, such as the cell cycle sequencing and the succession of the proliferative generations (flow cytometry with propidium iodide labeling and CFSE respectively - carboxy fluorescein diacetate imidil succinic ester), correlated in case of **Dermo-Dis** with determining the influence on collagen synthesis and activity of metalloproteinases.

The results were estimated as Proliferation Index, ie the sum of the percentages of cells in S and G2/ M phases of multiplication, calculated with a specific analysis software (FACS Express v3 module DNA cell cycle and proliferation).

Table 3. The effect of the compounds on the proliferative capacity of the cell line HS 27 (normal human dermal fibroblasts)

	medium containing normal estrogen levels				**medium without estrogen**			
	% S+ % G2/M	**% variation (%S+G2 /M)**	**P.I.**	**% variation PI**	**%S+ %G2/M**	**% variation (%S+G2 /M)**	**P.I.**	**% variation PI**
Celular control	26,73		3,04		24		2,82	
Solvent control	25,86	-3,25	3,27	7,57	35,8	49,17	2,85	1,06
Dermo-Dis 1nM	29,9	11,86	4,11	25,69	42,3	76,25	2,83	-0,70
Dermo-Dis 5nM	32,11	20,13	4,39	44,41	43,7	82,08	2,89	1,40
Dermo-Dis 10nM	30,7	14,85	3,05	0,33	55	129,17	4,62	62,11
Dermo Ska 1/8000	30,79	19,06	5,17	58,10	49,8	39,11	3,86	35,44
Dermo Ska 1/16000	29,71	14,89	3,01	-7,95	28,86	-19,39	4,47	56,84
Dermo Ska 1/32000	27,83	7,62	3,18	-2,75	28,03	-21,70	2,19	-23,16
Dermo Sks 1/64000	26,24	-1,83	3,2	5,26	25,37	-29,13	2,72	-4,56
Dermo Sks 1/32000	25,51	-4,56	3,16	3,95	27,9	-22,07	2,6	-8,77
Dermo Sks 1/16000	25,8	-3,48	3,06	0,66	28,8	-19,55	2,4	-15,79
β estradiol 1nM	32,11	20,13	3,24	-0,92	35,61	48,38	3,91	38,65

Dermo- Dis 10nM and **Dermo-Ska** 1/8000 stimulates the cell proliferation rate at the keratinocyte level under estrogen-depletion, similar to the synthetic estrogen, β-estradiol. At the fibroblast level, estrogen-mimetic effect is maintained for both the **Dermo-Dis** (5 nM and 10 nM) and for **Dermo-Ska** (1/8000 and 1/16000) extract, the

two active ingredients showing concerted action at the estrogen skin receptors, similar to the synthetic products (β estradiol). The Dermo-SKs extract, containing hydrolyzed steroidal saponins, does not affect the proliferative activity in estrogen-depleted conditions, supporting the hypothesis of the estrogen-mimetic action of the steroid aglycon (Dermo-Dis) and the extract that contains the aglicon (Dermo - Ska) by binding to the estrogen receptors of the fibroblasts (Patent Application 00482, 2012).

Evaluation of the intracellular calcium and its influx under the action of the compounds with estrogen-mimetic activity, Dermo-Ska and Dermo-SKs is shown in Fig. 51 and Table 6, as a result of the experiment under the same conditions described for the action of Dermo-ET extract (the fibroblasts adhered for 48h, 48h treated with the substance of interest and then labeled with fluorophore FLUO 4 with FITC-A emission coordinates, acquisition and analysis by flow cytometry).

Table 4. Modulation of the intracellular calcium (expressed as median channel fluorescent emission FITC-A) under the effect of the compounds isolated from *Trigonela foenum-graecum* in culture media containing different estrogen levels.

	medium without estrogen		**medium containing estrogen levels**	
	FITC-A without ionophore	**FITC-A with ionophore**	**FITC-A without ionophore**	**FITC-A witho ionophore**
Control	112	187	285	392
Solvent Control	113	181	278	397
DERMO Ska 1/8000	172	228	363	429
DERMO Ska 1/16000	166	201	257	402
Dermo-Dis 1nM	168	211	349	415
Dermo-Dis 5nM	154	289	243	385
17 β estradiol	306	455	180	381

Both **Dermo-Dis**, as well as extract containing the steroid aglycone **Dermo-Ska** acts to increase intracellular calcium concentration through both as flow from the extracellular environment and the mobilization of the intracellular deposits. Dermo Dis 1nM and Dermo-Ska 1/8000 enhances the activity of ionophore A23187 in culture medium with normal estrogen levels, but in case of the estrogen depletion, **Dermo-Dis** 5nM is the most active compound in accelerating the flow of calcium ions from the extracellular environment into the intracellular one. However, the activation of the cell proliferation, especially in estrogen-depleted conditions is performed by **Dermo-Dis** and the equivalent extract **Dermo-Ska** through signaling pathways that involve the activation of the calcium channels and its involvement as a secondary messenger.

Modulation of the signal molecules involved in skin firmness (α1β1 and α2β1 Integrines, TGF-β) by compounds isolated from *Trigonella foenumgraecum* and *Trifolium pratense*

The stimulation of TGF β release, key factor of the balance between collagen synthesis and activation of extracellular matrix metaloproteinases

We tested some active principles capacity to modulate extracellular secretion of TGF-β, as a part of the estrogen depletion experimental model, in order (DUMITRIU et al., 2013). We choose the substances for tests based on our previous results concerning **Dermo-ET** estrogen- like action, as well as those of steroidic aglicon **Dermo-Dis** and *Trigonella foenumgraecum extract* containing this aglicon (**Dermo-Ska**). We tested:

a) **Dermo-ET** compared with compounds from its composition: daidzeina, genisteine, biochanine and formononetin.

b) **Dermo-Dis** and **Dermo-Ska**

The results are presented in the following tables and expressed, in ng/ml TGF-β:

a) Dermo-ET from *Trifolium pratense*.

Table 5 . TGF-β evaluation in culture media with different estrogenic content

	Normal estrogenic content		Lack of estrogens in culture media	
	TGF beta (ng/ml)	**% of variation**	**TGF beta (ng/ml)**	**% of variation**
Control	1420,8		1411,74	
PG	1435,37		1345,37	
Dermo-ET 1/1000	1441,96	7,2	1588,01	18,1
Dermo-ET 1/2000	1339,34	-0,4	1213,06	-9,8
Daidzeina Dermo-ET 1/1000	1417,78	5,4	1448,01	7,7
Daidzeina Dermo-ET 1/2000	1231,06	-8,5	1460,11	8,6
Genisteina Dermo-ET 1/1000	1387,59	3,2	1788,6	33,0
Genisteina Dermo-ET 1/2000	1441,96	7,2	1472,21	9,5
Biochanina Dermo-ET 1/1000	1363,46	1,4	1417,78	5,4
Biochanina Dermo-ET 1/2000	1270,12	-5,6	1407,33	4,6
Formononetin Dermo-	1405,7	4,5	1666,51	23,9

ET 1/1000				
Formononetin Dermo-ET 1/2000	1387,59	3,2	1764,14	31,2
(D+G+B+F) echiv Dermo-ET 1/1000	1429,87	6,3	1675,65	24,6
(D+G+B+F) echiv Dermo-ET 1/2000	1484,32	10,4	1563,13	16,2
β estradiol 1nM	1605,66	19,4	1535,83	14,2

Dermo-ET stimulate the cellular proliferation rate of fibroblast in normal condition of cellular development, as well as in estrogen depletion case, similar with the synthetic estrogen, β-estradiolul. β estradiol acts in an unspecific manner, stimulating the TGF-β production, conducting to an uncontrolled collagen synthesis leading to fibrosis. **Dermo-ET** acts in a specific manner, only in estrogen depletion conditions, when the collagen synthesis is lower, assuring this way the proteic structure of the dermis. The estrogen-like effect is proved by each component of isoflavones mixture, especially by genistein and formononetin.

b) Dermo-Dis and **Dermo-Ska** from *Trigonella foenumgraecum* effect on TGF-β release.

c)

Table 6. TGF beta release in culture media with different content of estrogens

	Normal estrogenic content		**Lack of estrogens in culture media**	
	TGF beta (ng/ml)	**% of variation**	**TGF beta (ng/ml)**	**% of variation**
Control	105,62		92,52	
Solvent control	104,54		91,22	
Dermo-Dis 1nM	148,02	95,5	118,68	28,2
Dermo-Dis 0,5nM	241,01	218,3	191,21	106,6
Dermo-Ska 1/8000	257,8	144,08	204,8	121,3
Dermo-Ska 1/16000	186,5	76,6	164,4	77,7
17 β estradiol 1nM	208,51	175,4	168,51	82,1

Dermo-Dis, especially the 0,5nM dose, as well as the extract that contain this steroidic aglicon (D**ermo-Ska**) stimulate TGF-β release, a correlative effect with cell proliferation proved in chapter 6.2. The effect is17 β estradiol like, directing the collagen synthesis process even in normal culture condition, that could generate the fibrosis

process. In estrogen depletion, these substances have a stimulatory effect on TGF-β release, important in protein structures relief from the extracellular matrix.

α1β1 and α2β1Integrines

Results from three succesive experimental series are shown in the following tables:

Table 7. α1β1 and α2β1integrines expression induced by Dermo-ET.

	FITC-A Mean (CD 49b - α2Inte-grine)	**% of variation**	**PE-A Mean (CD 49a -α1Inte-grine)**	**% of variation**	**APC-A Mean (CD 29 – Inte-grine β1)**	**% of variation**
CONTROL	14448		5837		4167	
SOLVENT CONTROL	15122	4,67	6330	8,45	4984	19,61
TGF-β 4ng/ml	44217	206,04	7005	20,01	8123	94,94
Dermo ET 1/1000	29921	107,09	4851	-16,89	3719	-10,75
Dermo ET 1/2000	25796	78,54	4092	-29,90	4314	3,53
Formononetin equivalent Dermo ET1/1000	14313	-0,93	3193	-45,30	2748	-34,05
Biochanina equivalent Dermo ET 1/1000	24491	69,51	3220	-44,83	3594	-13,75
Genisteine echivalent Dermo ET 1/1000	23269	61,05	3834	-34,32	4448	6,74
Daidzeine equivalent Dermo ET 1/1000	18954	31,19	3797	-34,95	3597	-13,68
(Daidzeine+Genisteine +Biochanine+Formononetin) equivalent Dermo ET 1/1000	27371	89,44	6830	17,01	5308	27,38
(Daidzeine+Genisteine +Biochanine+Formononetin) equivalent Dermo ET 1/2000	13679	-5,32	6726	15,23	5489	31,73

The tests were shown the selective action of **Dermo-ET**, in dose / effect manner,

only on supraexpression of α2 glycoproteic chain, indicating an amplification of collagen of type I - fibroblast bounds and the stimulation of collagenase activity, acting on fibrilogenesis. We prove that this action is due to biochanine and genisteine especially. The effect is similar with those of positive control (TGF-β 4ng/ml).

Table 8. Integrines chains expression induced by *Dermo-Ska, Dermo-Sks and Dermo-Dis*

	FITC-A Mean (CD 49b - α2 Integrines)	% of variation	PE-A Mean (CD 49a - α1 Integrines)	% of variation	APC-A Mean (CD 29 - β1 Integrines)	% of variation
Control	14227,67		5310,67		4077,33	
Solvent Control	14526,33	2,10	5490	3,39	4170,33	2,28
TGF-β 4ng/ml	46264	225,18	7005	31,92	8123	99,22
Dermo Dis 5nM	16703	17,40	4433,33	-16,51	5025,33	23,25
Dermo Dis 10nM	23169	62,85	5601,33	5,49	5922,33	45,25
Dermo Ska 1/8000	24289,33	70,73	6088,67	14,66	5411,67	32,73
Dermo Ska 1/16000	22641,67	59,15	5345,67	0,67	5887,67	44,40
Dermo Sks 1/35000	10787	-24,18	4182	-21,24	3842	-5,77
Dermo Sks 1/50000	34526,5	142,68	7461,5	40,52	4456	9,29
Dermo Sks 1/60000	47230	231,97	7424	39,81	3718,5	-8,80

Dermo-Ska induce especially α2 glycoproteic chain over-expression, but **Dermo-Sks** is the most active compound in α1 and α2 chain expression, even more active than the positive control (TGF-β 4ng/ml). These results recommend the phytocompounds as active ingredients in restoring the firmness of the skin, consolidating cell- extracellular matrix bounds.

THE ANTIINFLAMMATORY ACTIVITY OF COMPOUNDS *AT VASCULAR ENDOTHELIUM LEVEL*

The aim of these experiments were to investigate the antiinflammatory effect with the following applications:

- **Fighting against inflammation and scaring in photo-ageing**
- **Fighting against inflammation and bacterian infection in acneic lesions.**
- Acting as **anti-edematous and vascular toning**.

The experimental models used in these investigation was focused on the following parameters:

I. Inhibition of monocyte - endothelium adhesion through flow cytometry studies of proteic expression of VCAM-1 and ICAM-1;

II. Flow cytometry evaluation of pro-inflammatory cytokines release by the stimulated endothelial cells.

III. Highlight the pro-angiogenic VEGF factor expression.

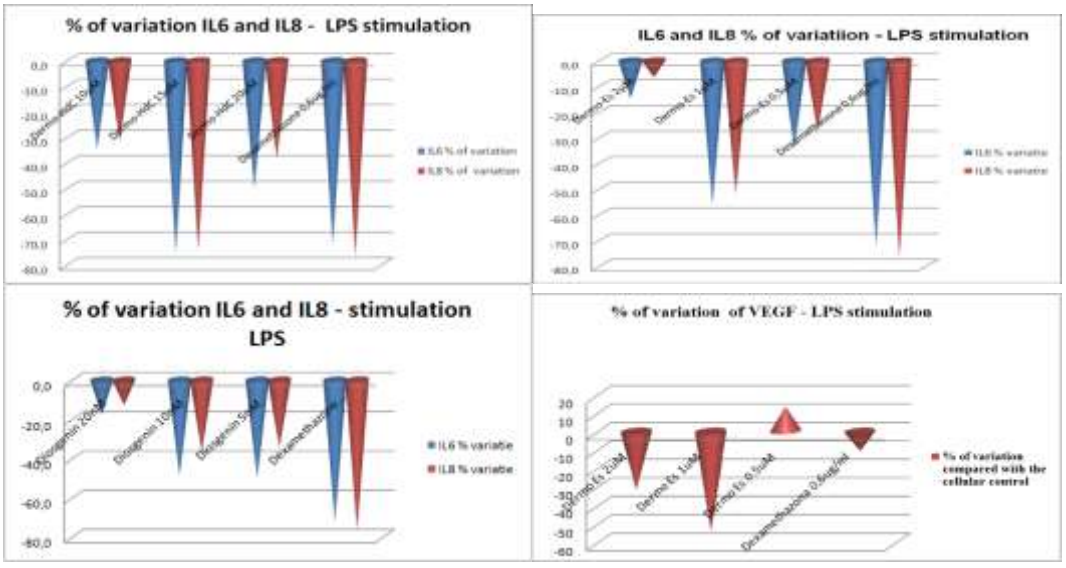
We performed 5 succesive experiments in order to settled the relevant stimulation conditions and the vascular effects of the following compounds:

a) **biocomplexes with high content of** *triterpenic saponines:* **DERMO-Es** and **DERMO-Hdc.**

b) **biocomplexes with high content of steroidic compounds:** *saponines* or *aglicones* **(Dermo SK-s, Dermo-Dis)**

The evaluation will be done compared with a positive control of Dexamethasone, a known antiinflammatory agent, 0,6µg/ml (Hettmannsperger et al., 1992; Wenchieh et al., 2002).

The experimental systems used consist in differentiated stimulation of endothelial cells with LPS, a lipopolisaccharide that mimics the bacterian infection, and with TNFα- an unspecific stimulus for systemic inflammation. LPS is a major component of gram negative bacteria acting as endotoxine for animals, inducing a strong immune response and producing pro-inflammatory cytokines.TNF-α take part from cytokines group that stimulate the acute phase inflammatory reaction. In order to settle the doses and incubation time for stimulation, we tested several variants starting from the relevant literature data for adhesion molecules and inflammatory cytokines (Dumitriu et al., 2013). The following figures present a selection of relevant results for the main effects we focused:



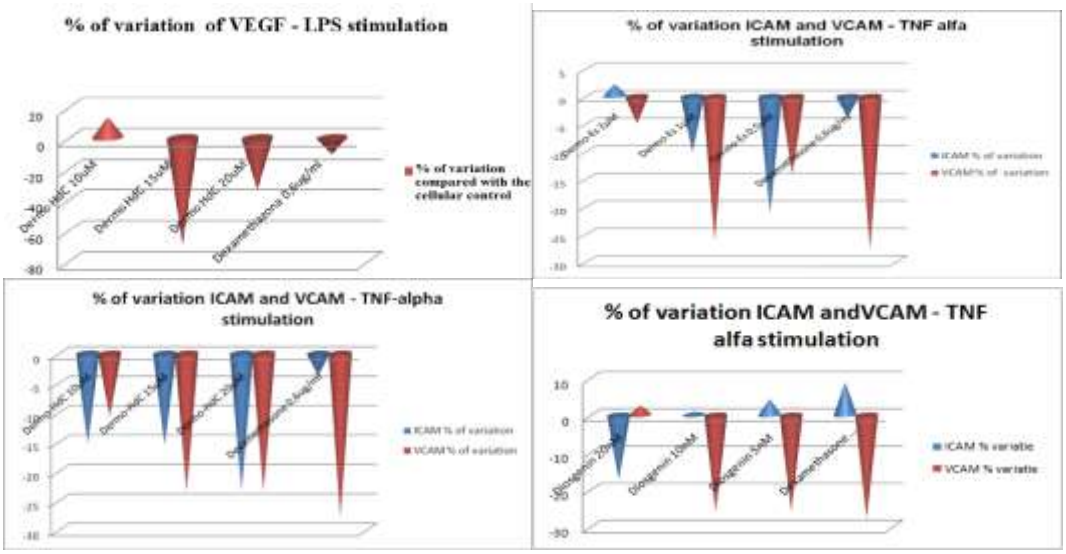


Figure1. Selection of results proving the vascular antiinflammatory effect of Dermo-HdC, Dermo-Es and Dermo-Dis

Based on these experimental results we can describe the following configuration regarding the anti-inflammatory activity of compounds **Dermo-Es, Dermo-HdC** and **Dermo-Dis** (with + we symbolize the positive effect, stopping the inflammation on the signaling pathway studied; - represent the lack of a significant activity).

Table 9. The antiinflammatory action of Dermo-Es, Dermo-HdC and Dermo-Dis

Bioactive compound/ / cellular parameter	**LPS stimulation (bacterian inflammation))**			**TNFα stimulation (Systemic inflammation, different etiologies)**		
	Adhesion molecules (ICAM)	pro-inflammatory cytokines IL6 andIL8	Vascular permeability (VEFG)	Adhesion molecules (ICAM andVCAM)	pro-inflammatory cytokines IL6 andIL8	Vascular permeability (VEFG)
Dermo-Es	-	+	+	+	+	-
Dermo-HdC	-	+	+	+	+	+
Dermo-Dis	+	+	+	+	+	+

The studied compounds had a different action, dose, as well as signal pathway dependent. For a complete *in vitro* screening regarding the antiinflammatory effect, it must have in attention several cellular processes that converge to a similar therapeutical target. The cytokines inhibition (IL6 şi IL8) represents a synergic effect both on the neutrophiles activation process (including enzymatic granules release that degrade the connective tissue) and on inflammation progression from acute level to the chronic one. This stage is accompaigned by VEGF inhibition correlated with the decrease of vascular

permeability and edemas, as well as the regulation of over-expression of adhesion molecules (ICAM and VCAM) triggered as one of the first response of endothelial pro-inflammatory stimulation.

The Inflammation triggered by the bacterial stimuli is inhibited by the action of both compounds by blocking the secretion of the IL6 and IL8 cytokines and the pro-angiogenic factor VEGF, which involves stopping the extracellular pro-inflammatory signaling cascade and reduce the vascular permeability. In this unspecific type of inflammation, the Dermo-Es and Dermo HdC action on the expression of the adhesion molecules is not significant (% variation below 10%).

During stimulation with TNF-α, it was demonstrated the concerted effect of Dermo-HDC in a dose-dependent manner to inhibit IL6 and IL8 secretion,, ICAM and VCAM expression and VEGF reduction factor. Dermo-Es acts in only two of the three signaling pathways investigated, decreasing of pro-inflammatory cytokines and inhibition of ICAM and VCAM expression.

Dermo–Dis has an antiinflammatory effect, inhibiting IL6 and IL8 release during the TNF-α unspecific stimulation, and as well as the expression of ICAM adhesion molecule (one of the firsts processes at the inflammation initiation). The active doses are between 5nM and 20nM. Dermo-Dis acts as well as in un-stimulated conditions on the initiation of vascular angiogenesis (important in scare healing) - rise the VEGF expression in extracellular culture medium. In unspecific stimulation conditions inhibits VEGF, acting on angiogenesis and vascular permeabilisation reduction.

The excessive activation and functional abnormalities of endothelial cells are associated with important pathologies (atherosclerosis, inflammation and cancer), as well as skin diseases (venous ulcerations, wound healing).Our results can be the basis for explaining the mechanisms of action and therapeutic application in the field of vascular inflammation. (patent application A00618, 2012).

CUMULATED CELLULAR PROTECTION AGAINST UV RADIATION INDUCED BY VEGETAL EXTRACTS FROM *TRIFOLIUM PRATENSE, TRIGONELLA FOENUMGRAECUM, TRITICUM AESTIVUM, MEDICAGO SATIVA AND CRATAEGUS MONOGYNA.*

Considering the major pathways for photo-ageing pathogenesis, we focused our screening on the following cell parameters: **Induction of apoptosis**; disturbances of **Cell cycle sequentiation**, **Cellular oxidative stress** - activated intracellular reactive oxygen species; Inflammatory Status: **secretion of pro-inflammatory cytokines** (IL6, IL8 , TNF-α); **IL1-α** as an indicator of irritability; **Secretion of VEGF** factor (vascular endothelial growth factor) - promoter of angiogenesis and the key step in restoring the damaged skin tissue and wound healing.

The keratinocytes were allowed to adhere for 24h, pretreated with the test substances for 24h., specific UV doses irradiated and then cultivated another 16h. with the tested substances. The irradiatin with specific doses used a controlled system: Bio-Sun (Vilber-Lourmet) Irradiation doses were established after preliminary tests as following: **UV-A – 9J/cm2; UV-B 0.02J/cm2,** values for which the cells maintain a 50% survival rate, but a strong pro-apoptotic effect. (Dumitriu et al., 2013).

The experimental design consist of 3 sample series, as following: **Series I:** - unexposed cells (control group); **Series II**- UV-A irradiated cells; **Series III:** UV-B irradiated cells.

The following pictures presents a selection of the significant results regarding the UV cellular protection induced by the bioactive compounds:

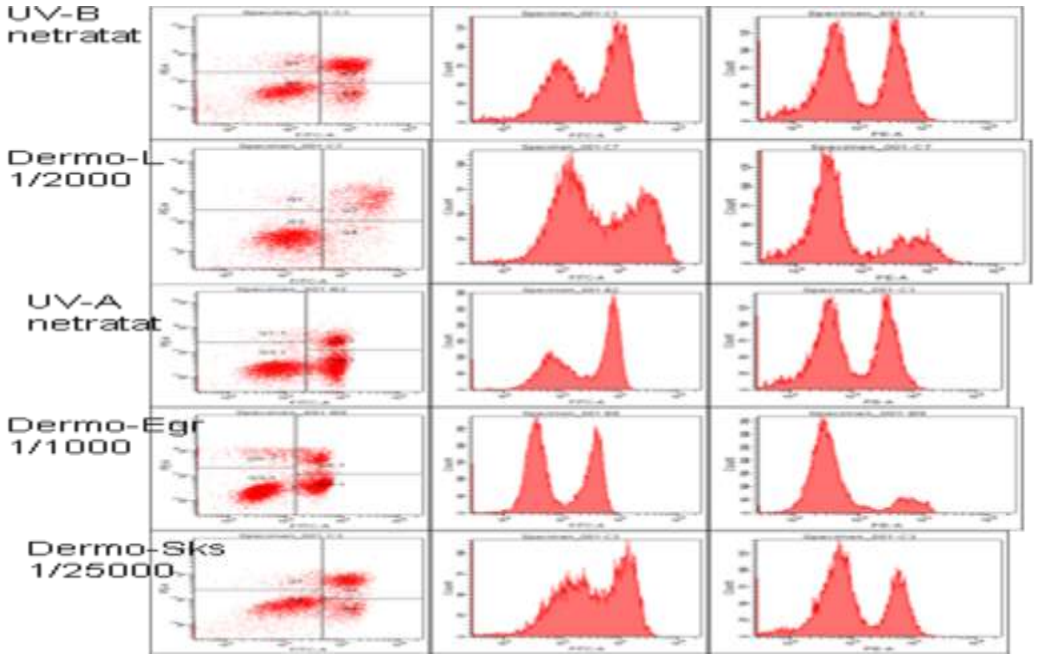


Figure 2. Flow cytometry diagrams presenting apoptosis protection induced by tested compounds.

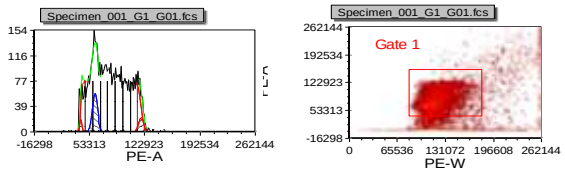
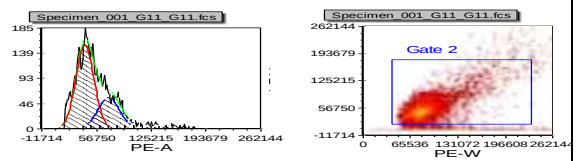
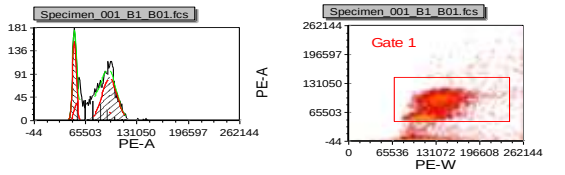
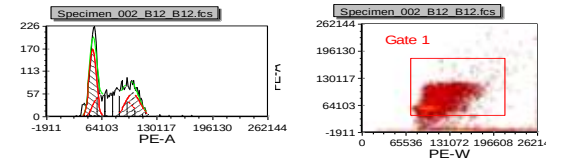
	**Interpretation** MultiCycle suggestions (a guideline only): An aneuploid DNA content is observed. The average %S=89.5, %G2=2.59 The S Phase confidence is good **Experiment Statistics** Chi sq: 2.73 Average %S: 89.54 Average %S aneuploid: 36.35 BAD: 1.17 Number of cells: 6335.00 Number of cycles: 2.00 Cycle fit model: 2 Cycle	UV-B irradiated cells
	**Interpretation** MultiCycle suggestions (a guideline An aneuploid DNA content is observ The average %S=11.4, %G2=2.38 The S Phase confidence is good **Experiment Statistics** Chi sq: 4.16 Average %S: 11.40 Average %S aneuploid: 17.74 BAD: 0.00 Number of cells: 7051.00 Number of cycles: 2.00 Cycle fit model: 2 Cycle	UV-B irradiated cells treated with Dermo-P
	**Interpretation** MultiCycle suggestions (a guideline only): No abnormal DNA content is observed. The diploid %S=36.1, %G2=43.1 The S Phase confidence is fair Note: inter-model error, **Experiment Statistics** Chi sq: 1.90 BAD: 0.00 Number of cells: 4622.00 Number of cycles: 1.00 Cycle fit model: 1 Cycle	UV-A irradiated cells
	**Interpretation** MultiCycle suggestions (a guideline only): No abnormal DNA content is observed. The diploid %S=46.8, %G2=22.1 The S Phase confidence is good **Experiment Statistics** Chi sq: 1.58 BAD: 0.00 Number of cells: 5557.00 Number of cycles: 1.00 Cycle fit model: 1 Cycle	UV-A – irradiated cells treated with Dermo-P

Figure 3. Dermo-P biocompound effect on cell cycle progression – selection of significant results

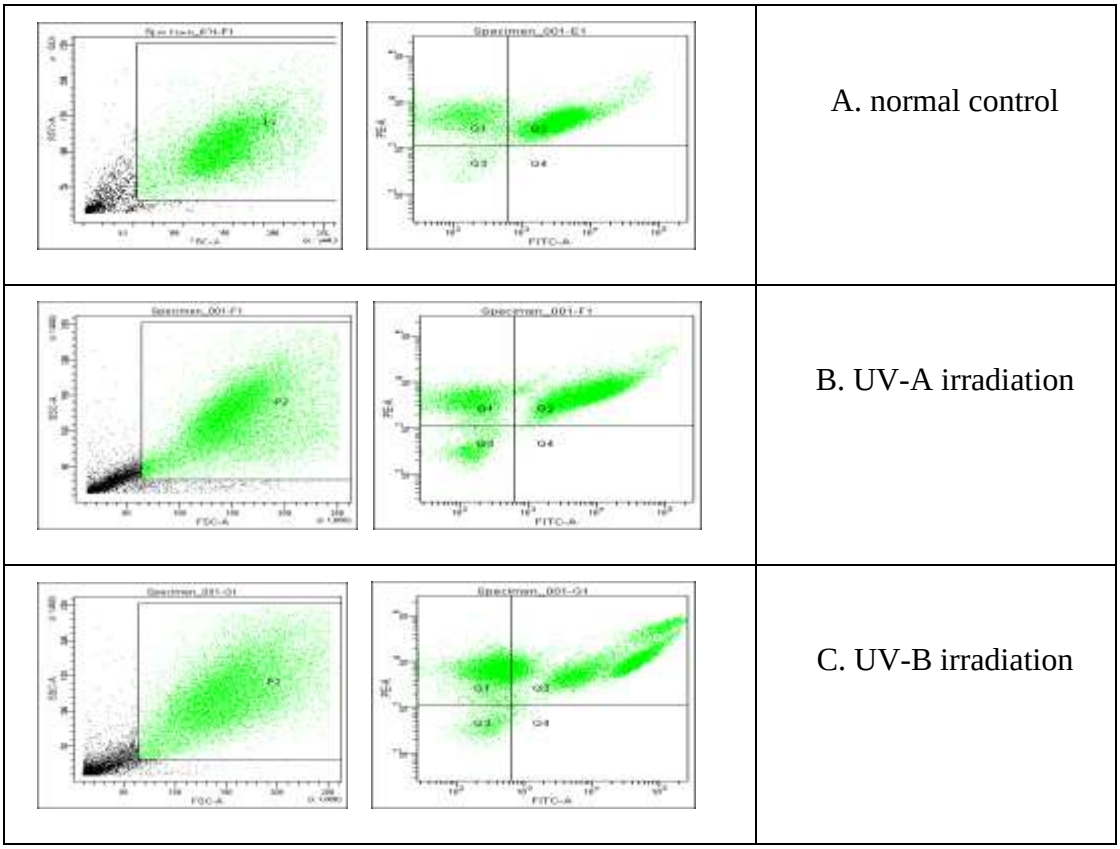


Figure 4. Flow cytometry diagrams showing cellular subpopulation producing hydrogen peroxide (FITC-A positive) and superoxide anion (PE-A positive). We notice the rising of hydrogen peroxide production (more cellular subpopulations stained in FITC-A coordinates), especially at UV-B irradiation.

The main screening results are presented in table 10 in order to highlight the most important signaling pathways as response to UV radiation exposure, as well as cumulative effects of bio-extracts.

Table 10. The main target pathways for the photo-protective action of the phyto-compounds

Tested extract / Biological extract	UV-A irradiated cells					
	Anti-apoptosis agents	**Cell cycle regulation**	**IL1α Inhibition**	**VEGF Inhibition**	**IL6 / IL8/IL10 / TNFα Inhibition**	**Oxidative stress inhibition (H_2O_2 and O_2^-)**
Dermo-Ska	no	no	yes	no	yes	yes
Dermo-Sks	yes	yes	yes	no	yes	yes
Dermo-L	no	yes	no	yes	yes	yes
Dermo-Egr	yes	yes	no	no	no	yes
Dermo-ET	yes	yes	no	yes	yes	yes
Dermo-P	yes	yes	no	yes	yes	yes

Tested extract / Biological extract	UV-B irradiated cells					
	Anti-apoptosis agents	**Cell cycle regulation**	**IL1α Inhibition**	**VEGF Inhibition**	**IL6 / IL8/IL10 / TNFα Inhibition**	**Oxidative stress inhibition (H_2O_2 and O_2^-)**
Dermo-Ska	no	yes	yes	yes	yes	yes
Dermo-Sks	no	yes	no	yes	yes	yes
Dermo-L	yes	yes	no	no	yes	yes
Dermo-Egr	no	yes	yes	yes	no	yes
Dermo-ET	yes	yes	no	no	yes	yes
Dermo-P	no	yes	no	no	yes	no

The specific screening of all target pathways reveals the complexity of cellular photo-protective mechanisms, allowing many chemical structures (triterpenic or steroid saponines, polyphenols, flavones, etc) to interact for the UV damages prevention. The bioactive extract with a free steroid aglycone (**Dermo-Ska)** had a protective effect against UV-A radiation with a complex action; stopping apoptosis induction and inflammatory cytokines IL6 and IL8 release, as well as IL1α. At UV-B exposure has only an anti-inflammatory and anti-angiogenic effects and didn't act on apoptosis prevention. The extract with steroid

saponines (**Dermo-Sks)** is an anti-inflammatory agent for both type of irradiation completed by the anti-angiogenic activity at UV-B exposure. **Dermo-L** is a cellular protector (anti-apoptotic and anti-inflammatory effect) at UV-B exposure, but unsuitable for UV-A radiation due to its photo-toxic potential (the rise of IL1α). **Dermo-Egr** had a protective effect specific to UV-A radiation (anti-apoptotic, anti-oxidant and anti-inflammatory action).

Dermo-P has a strong antioxidant action against both UV-A and UV-B, reducing the oxygen reactive species, inhibiting IL8and regulating cell cycle sequentiation. The extract is a cellular protector against apoptosis and inhibitor of IL6 production only for UV-A radiation (Dumitriu et al., 2012).

The red clover extract, Dermo-ET has a cellular protection effect against apoptosis induced by UV radiation, stopping the inflammatory process triggered by IL6 and IL8 signaling, a strong antioxidant action on reactive oxygen species in the case of UV-A irradiation.

The vegetal extracts complementarily mechanisms of action recommend their association in order to obtain an effective photo-protection.

Conclusions

The accelerate developping of the dermato-cosmetic industry and the developing of a border science, the dermato-cosmetology, impose the redirection of the research from this field to a better approach of cosmetic ingredients phisiological mechanisms of action at cellular and molecular level. This will precisely define the target effects and potential interactions between components, with a strong impact on toxicity and specific action of the future products.

The previous research studies highlight the complexity of cellular processes that are the basis of skin functional status, their interdependence and strict regulation, as well as the ultimate findings regarding the active skin treatment based on proved effects of active compounds.

The contribution in this field concern the highlight of phisiological mechanisms modulation at cellular and signal molecules level, as well as perturbancies caused by intrinsec factors (eg. estrogen depletion) or extrinsic ones (UV radiation, inflammatory stimuli) cured by the action of phytocompounds with controlled composition.

The proving of biological effect of vegetal extracts were done at the target cell level, on standardized cell lines relevant for the studied mechanism.

A. **The dermal restitutive effect** (**cellular turn-over and restoring the skin firmness)** were tested on human dermal fibroblasts (HS 27), the main cellular type responsible for the synthesis of structure proteins directed by TGFβ, as well as integrins expressions, molecules that realize the cell-extracellular matrix

adhesion. We also tested the estrogen-like mechanism of action, the estrogen depletion appearing in ageing, with a high impact at skin level.

B. **The photoprotective effect was proved on human keratinocyte cell line,** HaCaT, the first layer of skin functioning as a gate to UV radiation.
C. **The antiinflamatory effect at vascular endothelium level** was highlight **on a specific cell line, HUVEC** (human umbilical vein endothelial cells)

A. At dermal level, and respectively fibroblast as target cell, we noticed the following:

- The proliferative status interdependence with the estrogenic level and the calcium influx;

- The complex action of **Dermo-ET**, estrogen-like, rising the proliferative capacity of fibroblasts using Calcium as second mesenger; The estrogen-like effect is proved by each component of this isoflavones mixture, especially daidzeine, formononetin and biochanine regarding the DNA synthesis and mithosis progression.

- Cell proliferation activation, especially in estrogen depletion conditions, achived by **Dermo-Dis** and the similar extract **Dermo-Ska t**hrough signaling pathways **involving calcium channels activation and its function as second messenger.**

- β-estradiol acts unspecific, stimulating TGFβ release, a process that could induce fibrosis through an uncontrolled collagen synthesis. **Dermo-ET acts specifically only in estrogen depletion,** when the collagen synthesis is lower, assuring the protein structures support. The estrogen-like effect is proved by each component of isoflavones mixture, especially genistein and formononetin that restore the TGFβ release. ,

- **Dermo-Dis** (0,5nM), as well as the extract that contain this steroidic aglicon (**Dermo-Ska**) stimulate the TGF-β release and amplify the cell proliferation. The effect is unspecific, similar to 17 β estradiol, directing the collagen synthesis process even when it is not a necessity for dermal structures.

- The tests regarding the membrane expression of integrines highlighted the **Dermo ET dose / effect action**, only on α2 glycoproteic chain expression, indicating the **amplification of fibroblast – collagen type I bounds and the stimulation of collagenase activity**. This action is due especially to biochanine and genisteine from extract composition, similar to the positive control (TGF beta 4ng/ml). **Dermo-Ska** induces especially α2 glycoproteic chain expression, but **Dermo-Sks** is the most active compound in α1 and α2 chain over expression, better than the positive control (TGF-β 4ng/ml). As a consequence, these extracts could be recommended as active ingredients in skin firmness restoring, consolidating dermal cell – extracellular matrix bounds.

B.Regarding the photoprotective action on human normal keratinocytes, the extracts had different effects depending of the start vegetal raw material, acting on others

pathways of degradation and inflammation triggering. **Dermo-Sks** had a protective effect against UV-A radiation, with a complex action, stopping apoptosis induction and inflammatory cytokines IL6 and IL8 release, as well as IL1α. At UV-B exposure has only an anti-inflammatory and anti-angiogenic effects and didn't act on apoptosis prevention. **Dermo-Ska** is an anti-inflammatory agent for both type of irradiation, completed at UV-B exposure by the anti-angiogenic activity. **Dermo-L** is a cellular protector (anti-apoptotic and anti-inflammatory effect) at UV-B exposure, but unsuitable for UV-A radiation due to its photo-toxic potential (the rise of IL1α). **Dermo-Egr** has only a protective effect to UV-A radiation (anti-apoptotic, anti-oxidant and anti-inflammatory action). The complexity of cellular photo-protective mechanisms imposes a precise screening of all target pathways. **Dermo-P** has a strong antioxidant action against both UV-A and UV-B, reducing the oxygen reactive species, inhibiting IL8and regulating cell cycle sequentiation. **Dermo-ET** has a cellular protection effect against apoptosis induced by UV radiation, stopping the inflammatory process triggered by IL6 and IL8 signaling, a strong antioxidant action on reactive oxygen species generated by UV-A irradiation The vegetal extracts complementarily mechanisms of action recommend their association in order to obtain an effective photo-protection.

C. Regarding the ***in vitro*** **screening concerning the vascular endothelium antiinflammatory effect**, it was investigated different cellular processes, convergent to the same therapeutic target. The inhibition of both IL6 and IL8 represents a synergic process blocking the neutrophils activation (including the release of enzymatic granules that degrade the connective tissue), as well as stopping the inflammation progression from acute to chronic stage. This process is accompanied by VEGF inhibition, correlated with the decrease of the vascular permeability and reducing of oedema, as well as the normalisation of monocyte-endothelium adhesion molecules (ICAM and VCAM) over-expression, triggered as one of the first responses on endothelial pro-inflammatory stimulation. The Inflammation triggered by the bacterial stimuli is inhibited by the action of both compounds by blocking the secretion of the IL6 and IL8 cytokines and the pro-angiogenic factor VEGF, which involves stopping the extracellular pro-inflammatory signaling cascade and reduce the vascular permeability. In this unspecific type of inflammation, the Dermo-Es and Dermo HdC action on the expression of the adhesion molecules is not significant. During stimulation with TNF-α, it was demonstrated the concerted effect of Dermo-HDC in a dose-dependent manner to inhibit IL6 and IL8 secretion, ICAM and VCAM expression and VEGF reduction factor. Dermo-Es acts in only two of the three signaling pathways investigated, decreasing of pro-inflammatory cytokines and inhibition of ICAM and VCAM expression. **Dermo–Dis** has an antiinflammatory effect, inhibiting IL6 and IL8 release during the TNF-α unspecific stimulation, and as well as the expression of ICAM adhesion molecule (one of the firsts processes at the inflammation initiation). Dermo-Dis acts as well as in un-stimulated conditions on the initiation of vascular angiogenesis (important in scare healing) - rise the VEGF expression in extracellular culture medium. In unspecific stimulation conditions inhibits VEGF, acting on angiogenesis and vascular permeabilisation reduction.

Our studies have a particular scientific importance according to the fundamental

and applied research, making a worthy contribution to a new field of research: Dermato-cosmetology, by correctly defining the molecular/ cellular targets for a series of nine bioactive compounds. The impact of this research will affect the development of new *in vitro* screening methodologies, at the level of mechanism of action, based on the complex and interdependent evaluation techniques for proving dermato-cosmetics efficacy, as well as the configuration of better products.

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