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ANTI-ACNE PRODUCT DEVELOPMENT BASED ON VEGETAL EXTRACTS WITH ANTI-INFLAMMATORY AND DERMAL RESTORING EFFECTS

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Abstract

Developing a product designed for acne therapy had as a main objective the association of vegetal active principles joining mollecular and cellular targets, as following: Radix bardanae and Centaurea cyanus extracts for the anti-inflammatory effects at skin level, Trifolium pratense and Calendula officinalis for dermal reconstruction after acne scars.

The anti-inflammatory effects of Radix bardanae and Centaurea cyanus extracts were tested on HUVEC endothelial cell line, stimulated with LPS, a bacterial stymulus that mimics the acne developping conditions. The extracts inhibit the IL6 and IL8 pro-inflammatory cytokines and ICAM overexpression, mollecular events characterising the begining of inflammatory response on small blood vessels.

The dermal restoring effect was previously proved on normal dermal fibroblasts (HS 27 standardised cell line). The Trifolium pratense extract induces the cell proliferation and the integrine α2 – glycoprotein chain over-expression, indicate an amplification of fibroblasts – type I collagen bounds and collagenase activity stimulation, with an important role in fibrillogenesis. The Calendula officinalis extract decrease the TGF-β release, leading to metalloproteinases from extracellular matrix activation, a key process in tissue remodeling through enzymatic digestion of elastine and collagen deposits from scars.

The tolerance and efficacy tests for the anti-acne product, designed based on these results, proved a good skin compatibility, the decrease of healing time of acne lesions, the decrease of sebumetry and an optimum skin moisture after 4 weeks of treatment (clinical studies done at Dermatology Clinic of Clinical Hospital Colentina on 56 volonteers).

Key words: acne therapy, anti-inflammatory effects, dermal regeneration, Radix bardanae extract, Centaurea cyanus extract, Calendula officinalis extract, Trifolium pratense extract

Introduction

In the area of modern cosmetics, the main objective is creation of a therapeutic approach to improve the skin status, targeting cells or even genes with special designed active ingredients. In this context, the development of a new

product had to be based on scientific research focused on dermo-epidermal mechanisms controlling the functionality of skin tissue homeostasis and the modulation of these processes by the specific compounds with pharmacological activity.

Dermatocosmetic design knows a huge change, especially due to modern formulation techniques sustained by complex studies regarding the bioavailability of active principles, as well as the conceptual changes regarding the phisiological effects of the products. Gradually, cosmetic products became " cosmeceuticals", products with a scientific design and chemical, phisical and medical standards.

The effective development of cosmeceuticals is sistematically addressing to several aspects: products vision, the form of the product, essential ingredients, critical ingredients, product performance, compliance with legislative requirements. (1)

The cosmetic product development is a multi-step process based on a strict algorithm and several checking points in order to obtain the optimum profile for skin irritancy/efficacy (fig.1).

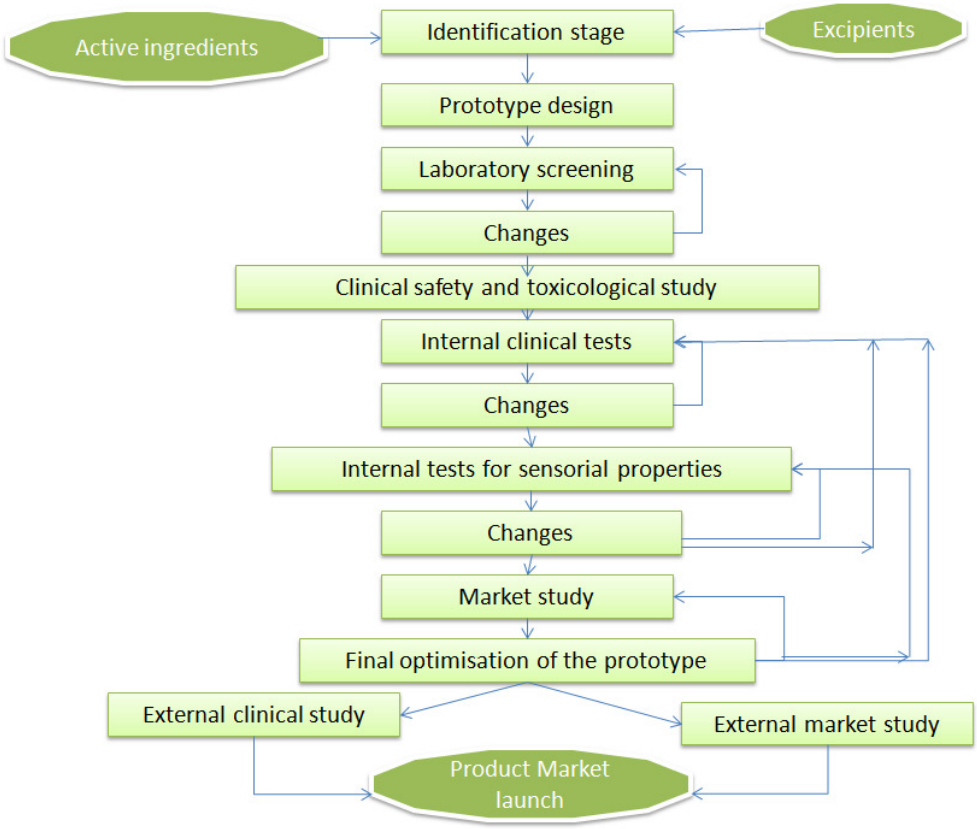


Fig. 1: Cosmetic product development algorithm for an optimum toxicity / efficacy profile. (1)

The first step in a new product design is the choice of active principles, in accordance with the therapeutical target proposed.

Acnee is a chronic, inflammatory disease of pilosebaceous glands characterised by macules, papules, pustules and nodules, sometimes cysts and scarring. The main factors involved in acne pathogenesis are: the rising sebum production, disturbance of bacterian microflora homeostasis, pilosebaceous unit cornification and inflammation. The skin and especially the pilosebaceous unit could be considered an endocrine organ, target for different hormones, with specific receptors expression.[2]. Androgen hormons supra – production and the lower level of estrogens are the basis for acne trigering, androgenic alopecia and seborrhea. The "skin hiperandrogenism" is the base for "in situ" over-expression of androgenic enzymes and for the hyper-responsiveness of the androgen receptors. [3]

Starting from traditional phyto-therapy data, the use of vegetal chemoprevention became of interest in cosmetic and pharmaceutical industry.

As anti-acne products, we target the anti-inflammatory effect at skin level (especially on blood vessels endothelium) and the dermal reconstruction after acne scars.

The most important point for a performant cosmetic product design is that "in vitro" cellular screening techniques, comprising an assembly of comparative and correlative methods oriented to prove the claimed effect and the specific target action of an ingredient, has to be projected on "in vivo" physiological phenomenon at tissue level. The implementation of a skin functional parameters scanning in respect of the active ingredients action will have a deep impact in the quality of final product formulation, a better treatment scheme for dermatological dysfunctions and rising performances for the new skin care ingredients.

One of the objectives of our studies is focused on **defining the main cellular processes according to the therapeutic target**, as follows: vascular endothelium level, with a major role in skin homeostasis by providing oxygen, nutrients and hormones (human endothelial cells) - **studies of inflammation of microbial origin**; and fibroblasts for skin reconstruction after scars – **the proliferative status, TGFβ signaling , molecular expression of the type α1β1 and α2β2 integrins**.

There are several aspects involved in vascular endothelium phisiology in accordance with inflammatory processes:

- the lymphocytes – endothelium adhesion, triggered by adhesion molecules expressed on cells membranes (VCAM 1- vascular cell adhesion molecule and ICAM 1 – intracellular adhesion molecule). Even both mollecules are over-expressed in inflammation, VCAM-1 control the start of

inflammatory process, ICAM-1 is expressed only in microvascular network.

- Pro-inflammatory cytokines IL6 and IL8, essential for endothelial activation, important in skin healing process. (4)

Dermal reconstruction after acneic scars could be accomplish throug several mechanisms:

- Fibroblasts proliferation important for the acceleration of cellular turn-over
- α1β1 and α2β2 integrins expression – surface cells receptors that mediate cells- extracellular matrix interactions.
- Modulating effects of the signal protein TGF-β, associated with tissue remodeling through enzymatic digestion of elastine and collagen deposits.

Cell proliferation and its correlations with several metabolic pathways, especially intracellular Calcium, is a relevant parameter for different skin pathological processes explanations. *(5).* Integrins – cell surface receptors controlling the informations transfer from inside to outside the cells - are involved in different cellular functions (growth and development, imune response and wounds healing. We design our studies on specific integrins with sustaining role for the dermo-epidermic tissue through cell adhesion to extracellular matrix proteins. α1β1 Integrine mediate the collagen synthesis feed-back, making connections collagen-cells or laminine 1 – cells. α2β1 Integrine mediate the type I collagenase (MMP1) stimulation with role in fibrilogenesis and collagen fibrilar organisation, conecting type I collagen. *(6)*

TGFβ is a chemotactic growth factor for fibroblasts, stimulating cell proliferation, rising prtein synthesis from extracellular matrix, haveing as main function the cell homeostasis regulation through its ability in changing the activation / inhibition ballance of matrix metaloproteinase (MMP) at genetic level. *(7)*

Another objective was **to develop therapeutic strategies** differentiated according to previously defined target mechanism specificity, based on the exploitation of some indigenous medicinal plants, in order to obtain classes of bioactive compounds with proven efficacy, eg. saponins, flavones and isoflavones, polyphenolcarboxylic acids, belonging to the following plant species: *Trifolium pratense, Radix bardanae, Centaurea cyanus, Calendula officinalis*.

We started our work from our previously communicated results regarding the following effects of the extracts:

1. **Dermo ET** accelerate the rate of fibroblasts multiplication, accompanied by the intracellular calcium mobilization, stronger in estrogen free culture medium, and induce the integrin α2 overexpression, indicating an increase in fibroblast - collagen type I connections and stimulating the collagenase activity with a role in fibrillogenesis. (8), (9)

2. Dermo - Oz complex from *Calendula officinalis* has a slow effect on cell cycle and proliferation, inducing a weak stimulation only after 48h/72h of action on fibroblasts culture. Dermo – Oz has an antifibrotic effect decreasing TGF-β secretion, associated with extracellular matrix metaloproteinases, important process in tissue remodeling through enzymatic digestion of imperfect fibrilar deposits. (10)

We shall improve the Dermo-Oz characterisation with intracellular calcium determination in conjunction with the proliferative status previously studied and integrines over-expression on fibroblasts. As well as, we shall complete our research with a cellular screening for Dermo-Br and Dermo-Abs anti-inflammatory effects on endothelial cells. Based on these "in vitro" results, we shall formulate anti-acneic products and tested them on human volonteers in order to prove their efficacy.

Materials and Methods

Cell cultures:

HUVEC - (humbilical vein vascular endothelium - ATCC® CRL-1730™). Cells were cultured in RPMI with 10% fetal bovine serum, 1% antibiotic / antimicotic solution, under standard culture conditions (37°C, 95% humidified air and 5% CO_2), harvested 24h before treatment, and 48 h with tested substances.

HS27 - (ATCC® CRL-1634™) – human normal foreskin fibroblasts. Cells were cultured in DMEM with 10% fetal bovine serum, 1% antibiotic / antimicotic solution, under standard culture conditions (37°C, 95% humidified air and 5% CO_2), harvested 48h before treatment, 48 h with tested substances.

Cells are harvested through tripsinisation (Tripsin/EDTA 0.1g% - Sigma).

Chemicals and reagents:

- FLUO-4 – AM – fluorescent reagent for intracellular calcium staining.
- Monoclonal antibodies for α1β1 and α2β1 integrins, BD cat. 559596, 555498, 559883 (**CD49a -PE**, for **α2**integrine ; **CD49b - FITC, for α1 integrine ; CD 29 - APC, for β1 integrine ; CD49e- PE**, for **α5** integrine).
- Monoclonal antibodies for ICAM-1 (APC Mouse Anti-Human CD54) and for VCAM-1 (PE Mouse Anti-Human CD106)
- BD Cytometric Bead Array (CBA)- Human Inflammatory Cytokines kit (BD Pharmingen)

Equipments:

- Flow cytometer FACS CANTO II with DIVA 6.1 and FCS Express softwares.

Methods:

a) Intracellular Calcium Evaluation: Calcium is an important second messenger involved in cellular processes, especially cell proliferation. The main signaling pathway of proliferative response are initialised by temporary rising of

intracellular calcium. We evaluated the intracellular calcium level by flow cytometry (fluorescent staining with FLUO-4-AM). We used calcium ionophore A23187 2,5µM as a control for cell responsiveness. Flow cytometry data were acquired in kinetics (dot plot representation Fluo4 fluorescence / time) and compared as fluorescence level (FITC-A) - FACS Diva software.

c) **Pro-inflammatory cytokines staining with *BD Cytometric Bead Array (CBA)* technique -** BD™ Cytometric Bead Array (CBA) is a flow cytometry application that allows users to quantify multiple proteins simultaneously. Each capture bead in the kit has been conjugated with a specific antibody. The detection reagent provided in the kit is a mixture of phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte (in our particular **case IL6 and IL8**). The analysis of the results (standard curve for each cytokine and concentration calculation) are done with FCAP Beads Array software. *(11)*

d) Endothelial adhesion molecules ICAM and VCAM - Cells are staining with specific fluorescent antibodies for ICAM-1 and VCAM-1: APC Mouse Anti-Human CD54 for ICAM-1 (intercellular-adhesion molecule); PE Mouse Anti-Human CD106 for VCAM-1 (vascular-cell-adhesion molecule). 10^6 cells are staining with 20µl antibody (BD Pharmingen), followed by incubation at room temperature, in the dark, 20 minutes. Cells are washed with 2ml staining Buffer (BD Pharmingen). The pellet is resuspended in 0.5ml Staining Buffer and the samples are ready to be acquired at the flow cytometer. The flow cytometer FACS CANTO II and FACS Diva 6 software are used to acquire and analyze "dot plot" representation for cell population visualization and fluorescence histograms for ICAM-1 (APC parameter), respectively VCAM-1 (PE - parameter). The results are evaluated comparing the mean of fluorescence channel APC or PE respectively.

e) **α1β1 and α2β1 integrins over-expression**:

staining with monoclonal antibodies to α and β chains (CD49a, PE fluorescent labeled, corresponding α2 subunit, CD49b, FITC fluorescent labeled, corresponding α1 subunit, and CD 29, APC fluorescent labeled, corresponding β1 subunit); flow cytometry analysis with DIVA 6 software.

Active compounds:

Radix bardanae extract (Dermo-Br), Centaurea cyanus extract (Dermo-Abs), Calendula officinalis extract (Dermo-Oz), Trifolium pratense extract (Dermo-ET) produced in Biotehnos laboratories.

Results and Discussion

A. "in vitro" antiinflammatory effect of Radix bardanae extract (Dermo-Br) and Centaurea cyanus extract (Dermo-Abs)

Endothelial cells (HUVEC cell line) were cultivated as described in chapter Matherials and Methods and **stimulated with 1µM LPS** (lipopolisaccharide that mimics bacterial infection, major component of gram negative bacteria, acting as endotoxine and inducing a powerful imune response, rising pro-inflammatory cytokines and adhesion mollecules expression). We compared our results with a well known antiinflammatory agent, **Dexamethasone 0.6µg/ml**, as a positive control.

A.1. ICAM and VCAM - Adhesion molecules expression on endothelial cells stimulated with LPS

One of the fundamental mechanism of inflammation progression is the leukocytic extravasation along the vascular endothelium and the infiltration in the adjacent tissue. *(12).* The infiltrate leukocytes secretes a high level of inflammation mediators, triggering the inflammatory response and resulting the injuries of the inflammed tissue. The trans-migration of the inflammatory cells along the microvascular endothelium is a decisive process mediated by adhesion mollecules express on the endothelial cell surface (ICAM and VCAM). In several dermatological pathologies the expression of these adhesion mollecules is significantly higher, assiciated with injuries of dermo-epidermic tissue and poor wounds healing.

Our experiments performed on an primary endothelial cell line (HUVEC) show a strong inhibition of both ICAM and VCAM induced by Dermo – Abs and Dermo –Br (Tabelno.1 and figure no.1) that will stop the migration of leukocyte at the inflammatory site and further the inflammation progression.

	Unstimulated cells				LPS 1µg/ml stimulated cells			
	ICAM (APC fluorescence channel median)	% of variation	VCAM (PE fluorescence channel median)	% of variation	ICAM (APC fluorescence channel median)	% of variation	VCAM (PE fluorescence channel median)	% of variation
Cellular control	10882,6		1366,9		13974,4		15697,5	
Solvent control	10436,0		1448,0		11789,0		11053,0	
Dermo Abs 1/2000	11773,0	8,2	1480,0	8,3	10841,0	-22,4	11058,0	-29,6
Dermo Abs 1/3000	11207,0	3,0	1319,0	-3,5	11766,0	-15,8	10422,0	-33,6
Dermo Br 1/1000	9896,0	-9,1	1422,0	4,0	10378,0	-25,7	10894,0	-30,6
Dermo Br 1/2000	10244,0	-5,9	1456,0	6,5	10096,0	-27,8	10439,0	-33,5
Dexamethasone 0,6µg/ml	10235,8	-5,9	1339,6	-2,0	11097,4	-20,6	13095,0	-16,6

Tabel no.1: Adhesion molecules ICAM and VCAM expression modulated by Dermo-Abs and Dermo-Br treatment

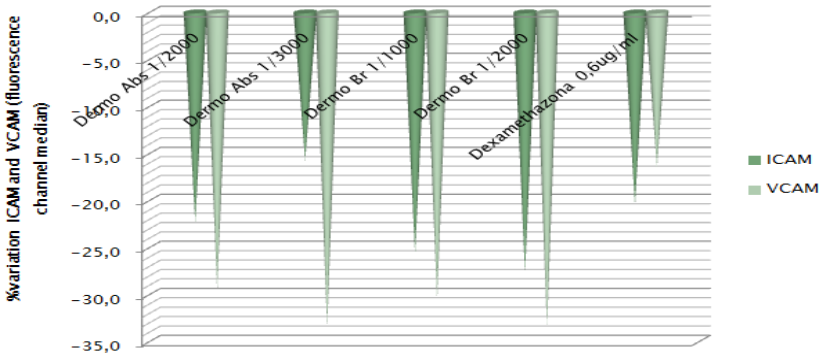


Figure no.1: % of variation of ICAM and VCAM expression modulated by Dermo-Abs and Dermo-Br treatment

A.2. IL6 and IL8 pro-inflammatory cytokines release from endothelial cells stimulated with LPS

IL-6 is a pro-inflammatory cytokine synthesised by monocytes and vascular endothelial cells, one of the main acute phase mediators. IL8 induces the neutrophils chemotaxis at the target cells. Therapeutical inhibition of IL6 and IL8 could initiate a potent anti-inflammatory action, their quantification at endothelial level indicating the efficacy of certain drugs. (13)

After 48h. of tested substances action, the treated cellular culture supernatant was stored at -70^{O}C for a further analysis. The soluble proteins IL6 and IL8 was quantified using CBA Inflammation kit, and fluorescent signal integration on a specific standard curve using FCAP Beads Array software. Results are presented in the tabel below (tabel no2)

	Unstimulated cells				LPS 1µg/ml stimulated cells			
	IL6 (pg/ml)	**% of variation**	**IL8 (pg/ml)**	**% of variation**	**IL6 (pg/ml)**	**% variatie**	**IL8 (pg/ml)**	**% of variation**
Cellular control	2054,5		2610,0		8166,1		4819,5	
Solvent control	2084,0		2497,5		7990,3		4626,7	
Dermo Abs 1/2000	1925,8	-6,3	2283,9	-12,5	5348,6	-33,1	2084,3	-56,7
Dermo Abs 1/3000	2195,6	6,9	2610,2	0,0	5558,2	-30,4	2353,6	-51,2
Dermo Br 1/1000	2369,4	15,3	3011,1	15,4	5746,5	-28,1	2539,2	-47,3
Dermo Br 1/2000	2103,1	2,4	2699,4	3,4	5630,3	-29,5	2260,6	-53,1
Dexamethasone 0,6µg/ml	1823,7	-11,2	1542,8	-40,9	2215,0	-72,3	1100,2	-77,2

Tabel no.2: pro-inflammatory cytokines release modulated by Dermo-Abs and Dermo-Br treatment

Dermo-Br and Dermo-Abs inhibits both IL6 and IL8 cytokines, blocking the neutrophyl activation, including the release of the enzymatic granules that digest the connective tissue, as well as stop the inflammation progression from acute to chronical stage. This process is accompaigned by the down-reglation of adhesion molecules expression, one of the first response of an inflammatory stimulus.

B. Intracellular calcium modulation - Dermo-Oz activity

The main signaling pathways required by the proliferative respose are transitory rising of intracellular calcium. The normal, dermal, human fibroblasts proliferation depends on extracellular calcium concentration. There are activators of this process on membranar receptors pathway, directing the calcium influx from extracellular environment to the intracellular one. *(14) (15)*

Fibroblasts were treated for 48h. with Dermo –Oz and Vitamin C, then harvested and stained with FLUO-4-AM. The cells responsivity regarding the transmembrane transport was checked with the ionophore A 23187, that open the calcium channels and sustain an influx of the ions from the outside to the inside of the cell if the calcium concentration is higher in the extracellular environment or in reverse sense if the intracitoplasmatic environment is rich in calcium ions.

Data were analised with Flow Jo software, before and after the addition of A 23187 (fig. 3).

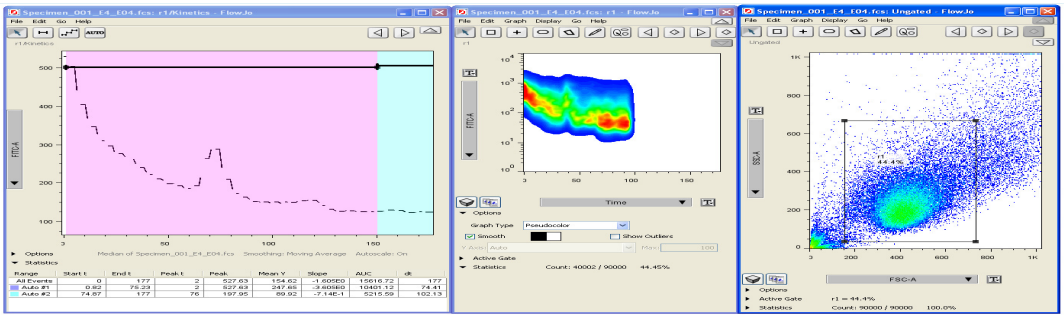


Fig. 3: Flow cytometry analysis of intracellular calcium (FLUO4-AM staining)

The graphic below (fig.4) presents the results of calcium mobilisation from intracellular compartiments induced by Dermo-Oz, compared with Vitamin C, a proliferation booster in fibroblasts (mean of three experiments).

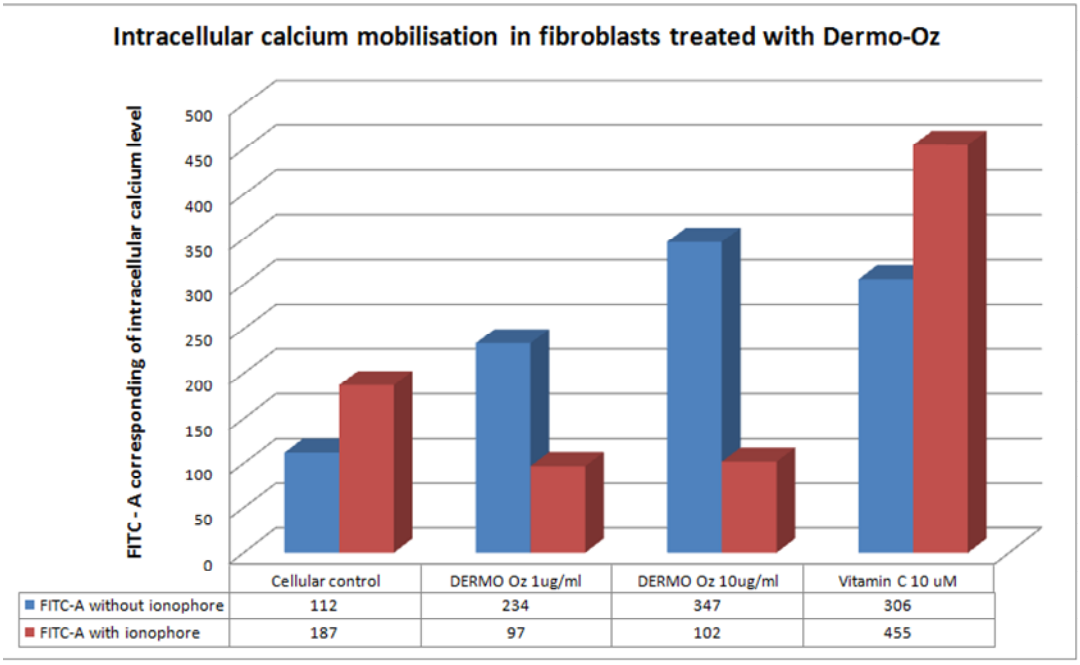


Fig. no. 4: Intracellular calcium mobilisation induced by Dermo-Oz

Data show a significant rising of intracellular calcium induced by **DERMO-Oz 10µg/ml,** similar with **vitamin C,** indicating a strong correlation with previously

communicated data regarding the boost of the proliferative status and TGFβ modulation induced by this compound. (10).

C. Integrins over-expression induced by Dermo-Oz in fibroblasts

Flow cytometry detection of integrins overexpression was performed on fibroblasts detached with Trypsin / EDTA (50% in phosphate buffer) in order to preserve the membranar glycoproteic domains. The cells were stained with specific antibodies as we describe in Materials and Methods chapter, running an isotypic control also, to assure the specificity of staining. Data from 3 succesive experiments are presented in the table below (tabel no. 3). Dermo-Oz action was compared with the positive control, TGFβ 4ng/ml.

	FITC-A Mean (CD 49b - Integrin alfa2)	% of variation	PE-A Mean (CD 49a - Integrin alfa1)	% of variation	APC-A Mean (CD 29 - Integrin beta1)	% of variation
Cellular control	14448,00		5837,00		5310,67	
TGF beta 4ng/ml	46264,00	220,21	7005,0	20,01	7005	31,92
Dermo Oz 5ug/ml	30387	110,32	5255,0	-9,97	5255	-1,04
Dermo Oz 10ug/ml	37317	158,28	6153,0	5,41	6153	15,88

Tabel no.3: Integrins expression on fibroblasts membrane induced by Dermo-Oz

Dermo-Oz 5 μM and 10 μM acts similar with TGF beta, only on α2 glycoproteic chain over-expression, indicating an **amplification of collagen type I – fibroblast connections and activation of collagenase with an importantant role in fibrilogenesis**.

Conclusions for "in vitro" studies:

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, as follow:

- **Radix bardanae extract (Dermo-Br) and Centaurea cyanus extract (Dermo-Abs)** - anti-inflammatory effect at vascular endothelium level,

through a concerted activity on IL6 and IL8 cytokines and adhesion molecules ICAM and VCAM.

- **Calendula officinalis extract (Dermo-Oz) –** antifibrotic effects (inhibits TGFβ, rise cellular proliferation in accordance with intracellular calcium mobilisation) (10), skin firmness effect through integrins over-expression
- **Trifolium pratense extract (Dermo-ET)** – estrogen-like effects, integrine α2 – glycoprotein chain over-expression, collagen biosynthesis activation - previously communicated results (8), (9)

Considering these results, we associated the extracts in order to accomplish **two anti-acne dermatocosmetic products** (cream and lotion) for hormonal balance modulation and scars prevention, based on estrogen –like and dermal reconstruction conjugated mechanisms.

EFFICACY STUDIES FOR THE ANTI-ACNE PRODUCTS:

The anti-acneic claim is a widespread effect for many skin care products, but usually a few test were done to scientific prove this action. In respect with new cosmetic regulations, we test our products efficacy through a clinical study performed in Clinical Hospital Colentina, Bucharest – Dermatology Clinic II.

The study involved 63 randomised patients with acne (53 women and 10 men), divided in 4 groups of test, in accordance with ethical principles of Helsinki Declaration concerning the rights, safety and comfort of participants. The test supposed 4 weeks of treatments and 5 points of monitoring the folowing parameters: **sebumetry, skin moisture and the change in percentage of acne lesions (papules, pustules, nodules),** compared with the starting point.

Results are presented in the folowing graphics (fig no.5 and fig. no.6):

Fig. no 5: Restoring of the skin phisiological parameters after the Anti-Acne Cream treatment

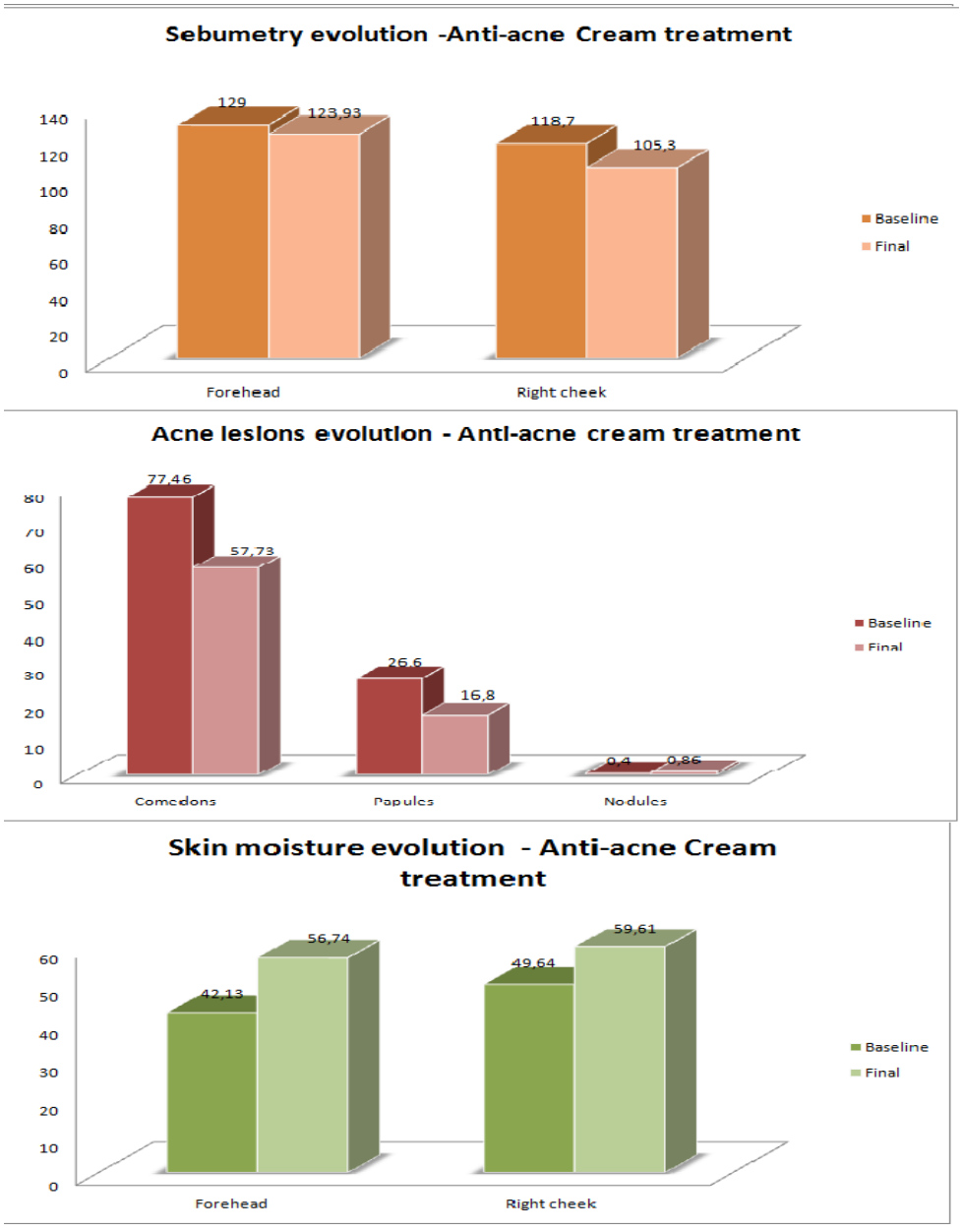
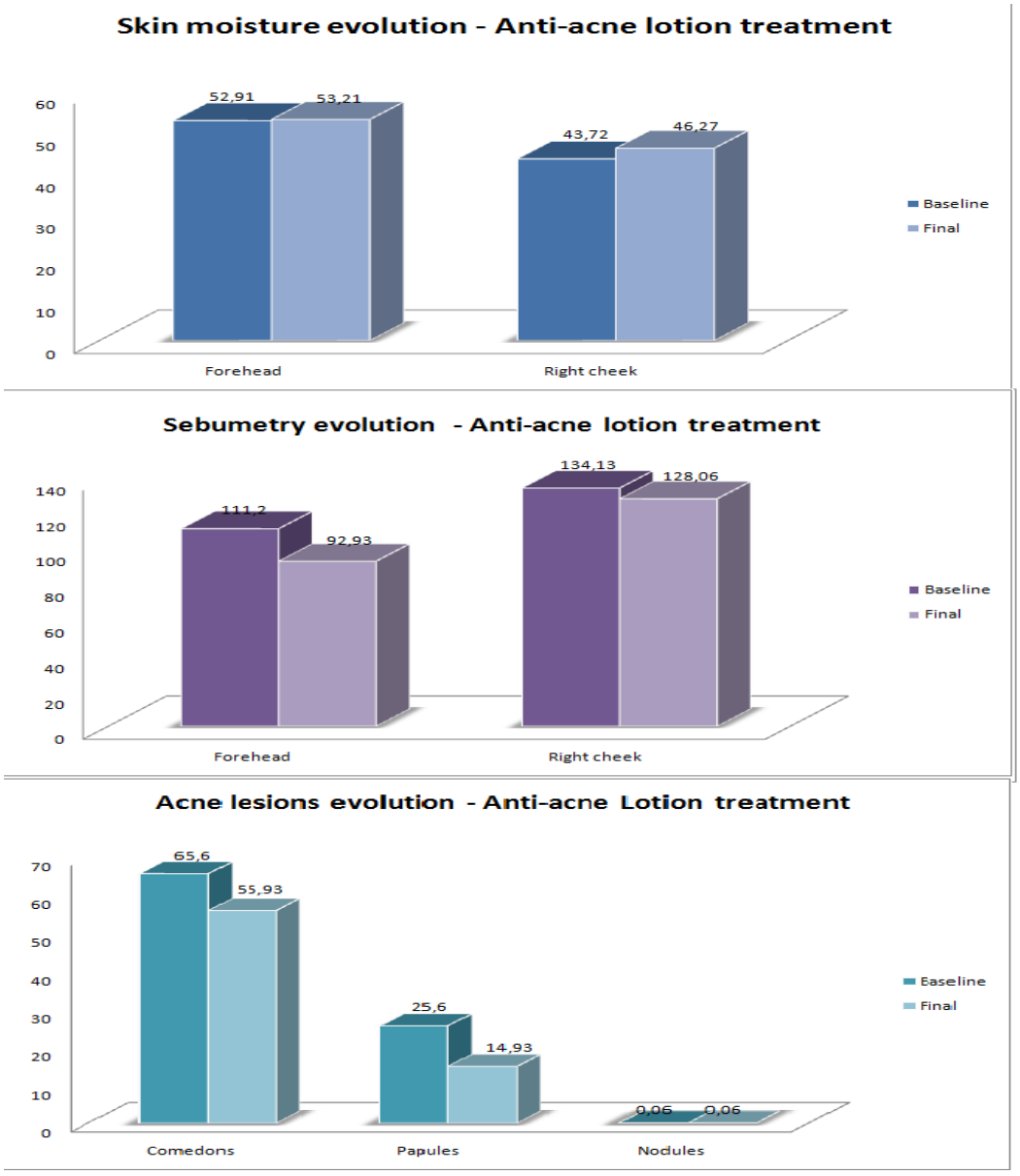


Fig. no 6: Restoring of the skin phisiological parameters after the Anti-Acne Lotion treatment



The anti-acne cream had a skin moisturising effect (25% - forehead region and 16% - cheek region), decrease the sebumetry especially in the cheek region (12%) and reduces the comedons (34%) and papules (58%). **The anti-acne lotion** is very active in anti-acne treatment, especially reducing the sebum (20% compared with the baseline) and reducing the number of comedons and papules (71%).

Conclusions

Despite the exponential develop of cosmetics with pharmaceutical activity, appropriates methods to quantify the effects are not yet established. We assist to an aggressive publicity of cosmetic products, which sometimes is not based on relevant investigations concerning the ingredients toxicity /efficacy profile, the cosmetic legislation being still permissive from this point of view. The experimental methods used in this study for defining the bioactive compounds mechanisms of action 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.

Our anti-acne products, designed upon a well doccumented cellular screening proving hormon-like, anti-inflammatory and anti-fibrotic effects, has shown significant "in vivo" action. The sebum quantity decrease in patients treated with anti-acne products and the skin moisture rise, inducing a good balance of skin phisiological parameters, especially after the cream treatment. The number of acneic lesions decrease, the majority of patients reported a better skin condition after the treatment, quicklier wound healing, with less scars.

Acknowledgment.

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