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DOCTORAL SCHOOL OF APPLIED SCIENCES
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SUMMARY OF THE PhD THESIS

ADVANCED RESEARCH FOR TESTING PLANT BIOCOMPLEXES ACTIVES ON SKIN DEGENERATIVE PATHOLOGIES

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CONTENTS

The summary of this thesis briefly presents the original results obtained, the general conclusions, the selective bibliography, as well as the scientific activity carried out during the doctoral studies, keeping the same notations used in the doctoral thesis.

OBJECTIVES AND PURPOSE OF THE STUDY

Oxidative stress, either exogenous or endogenous, is a biochemical trigger for the chain progress of physiological disorders involved in the pathogenesis of systemic degenerative diseases or on particular targeted tissue or organ level. The skin has a high exposure to external physical aggressions (eg UV radiation) and chemicals (eg xenobiotic agents), generating oxidative stress, becoming an organ susceptible to transformations generated by reactive free radicals, with repercussions in physiological changes, dysfunctions and pathologies induction: photosensitivity, burns, wounds and skin infections, malignant transformations. Disrupted skin homeostasis can be restored depending on the degree of imbalance by individual antioxidant intake, as well as by synergistic actions of some biologically active complexes interfering with mechanisms that generates the onset and / or progression of the disease (eg inflammation, cell proliferation, extracellular protein matrix recovery, reepithelialization, tumor progression) - **Figure 1**.

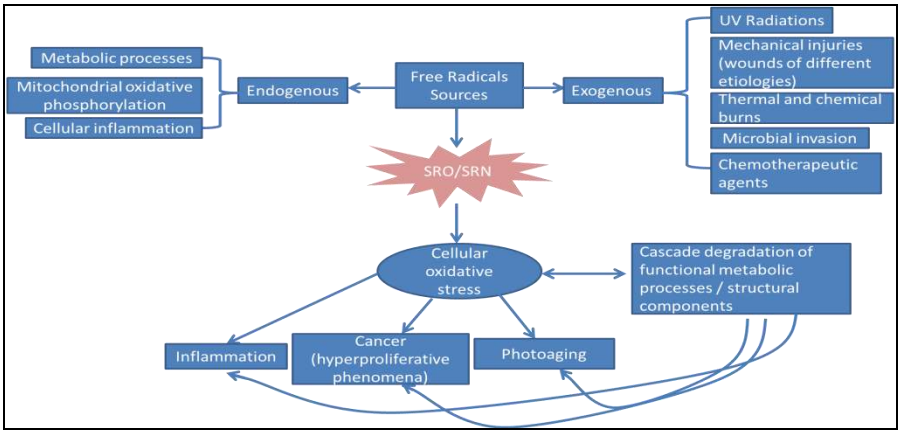


Figure 1. Triggers of cellular oxidative stress and its impact on degenerative processes

The purpose of the thesis research is the **identification, characterization, monitoring and *in vitro* modulation of cellular and molecular processes involved in skin degenerative disorders through the selective intervention of individual and associated biologically active complexes**. The variability of the cellular functional status will be mimicked *in vitro* by the signal molecules' actions which are recognized for their differentiated impact at the level of pro-oxidative and pro-inflammatory processes, ubiquity as spread and causality in degenerative pathologies.

The objectives of this study are:

- Development of *in vitro* experimental models, relevant for a complex multi-parametric screening of the plant biocompounds efficiency in dermatological therapies;
- Validation of experimental models by using known antioxidants (eg N-acetyl-Cysteine, vitamin C), anti-proliferative (methotrexate), or anti-inflammatory (dexamethasone) agents;
- Applicability of experimental models for defining the new product's effect, through the optimized plant biocomplexes' *in vitro* toxicological profile and specific action;
- Interrelated evaluation of active principles' effect ⟷ bioactive complex design and targeting the specific treatment of skin degenerative diseases.

The originality of this study consists in the obtaining applicability algorithm of biologically active complexes, with specific design and *in vitro* proved action in skin diseases, capitalizing the concept of "***green therapy***" on which the trends of the current cosmetics and pharmaceutical industry are based. Skin pathologies decrease the quality of life, having a major social and economic impact through the increased incidence at the population level, amplified by environmental and climatic conditions and the augmentation of pollution. Although there are multiple therapeutic solutions, especially synthetic ones, the effectiveness of the treatment decreases over time, due to the appearance of resistance phenomena or adverse allergic effects.

Although natural therapies have a traditional character, their scientific basis has not been fully clarified. In this context, this doctoral thesis has an innovative contribution on the approach and investigation of the optimal efficacy / toxicity profile for associated plant complexes in well-defined structural groups obtained in particular from the *Salvia officinalis, Arctium lappa, Corylus avellana, Trifolium pratense* extracts and waste plants of the *Solanum lycopersicum* and *Vitis vinifera* species.

Stages of action on the cell, *in vitro* determination and modeling of cellular status integrate culture techniques and advanced multiparametric screening methodologies at the cellular and molecular level in original

experimental models of irradiation, stimulation of specific growth factors and free radicals production cascades monitoring, using both standardized lines as well as normal and tumor primary cultures. A triggering factor of skin dysfunctions and development in degenerative pathologies is oxidative stress, which is the therapeutic target of the present study and the relevance of the multivalent efficacy of biocomplexes optimized in structure and effect.

Another important aspect of the researches, which emphasizes the innovative contribution, is the valorization of vegetable waste - **recovery and technological exploitation** of *Solanum lycopersicum* and *Vitis vinifera* species. These results: the convergence of research findings for the biological effects of the active extracts combinations have authentic perspectives in the formulation and conditioning further products for skin diseases.

Keywords: oxidative stress, skin tissue, photoprotection, inflammation, melanoma*, Salvia officinalis, Corylus avellana, Arctium lappa, Trifolium pratense, Solanum lycopersicum, Vitis vinifera*

The doctoral thesis entitled "*Advanced research for testing plant biocomplexes with action in skin degenerative pathologies*", consists of two parts and is structured in 5 chapters.

Part I. Current state of knowledge. The first three chapters present literature data, which reflect the current state of knowledge on the role of oxidative stress as a trigger in skin diseases, the impact of endogenous and exogenous factors generating oxidative stress, regulation of redox homeostasis by antioxidant intake.

Part II. Personal contributions. It highlights the action of some plant complexes, active in oxidative stress combating on the skin.

Chapter 4 presents the experimental models and methods used in their development, the studied plant extracts, as well as standardized cell lines (fibroblasts - HS27, keratinocytes - HaCaT, endothelial cells - HUVEC, murine melanoma cells - B16F10) relevant for the intended therapeutic effect. All reagents and equipment used in the experimental models are also presented.

Chapter 5 presents the highlighting of representative cellular and molecular processes for degenerative dermatological pathologies, modulated by natural active principles with antioxidant potential, through the development and application of experimental models.

PART I: THEORETICAL CONSIDERATIONS - CURRENT STATE OF KNOWLEDGE

Skin tissue is an important interface for maintaining homeostasis against mechanical, chemical and biological factors, faced by the external environment [Kolarsick et al., 2009]. The processes included in this category refer to: inflammation, immune response (including tolerance induction and disease prevention), wound healing and angiogenesis [Jia et al., 2015; Matthew A, 2018]. Epidermal and dermal cellular structures in the composition of skin tissue make a significant contribution not only to maintaining tissue homeostasis, but also to wound healing and underlie heterogeneity and tumor progression [Rogoni et al., 2018].

Oxidative stress is the result of disturbing the balance between prooxidants and antioxidants, being mediated by free radicals (ROS / RNS) generated during aerobic physiological metabolism and pathological inflammatory processes. The skin has an extremely complex antioxidant system that includes enzymatic antioxidants (glutathione-peroxidase, glutathione-reductase, superoxide-dismutase, and catalase) and non-enzymatic antioxidants (ascorbic acid, glutathione, uric acid, vitamin A, melanin, α-tocopherol, carotenoids, etc.) [Baek et al., 2016]. At the skin level, oxidative stress contributes to disorders such as loss of skin elasticity, photoaging, inflammation, autoimmune reaction, hypersensitivity, irregular keratinization, preneoplastic lesions and skin cancer [Nachbar et al., 1995]. Air pollutants, UV irradiation, microorganisms, viruses and xenobiotics can serve as exogenous sources of reactive oxygen species, while endogenous reactive oxygen species are generated during normal cellular metabolism, during immune reactions and several pathological diseases [Trouba et al., 2002; Portugal et al., 2007], such as the postischemic ones, in which reperfusion leads to a massive production of superoxide radical [Epstein et al., 1985]. ROS generated on the skin can be constitutively produced in epidermal keratinocytes by specific processes, such as enzymatic oxidation and aerobic respiration. ROS can also be induced by cytokines, growth factors and other physiological stimuli [Fuchs et al., 2001; Kohen et al., 2002].

The pathogenicity of skin diseases involves complex physiological, immunological, genetic, phenomena generated by two main agents (oxidants and cytokine network), which are involved in various skin disorders, including carcinogenesis, damages caused by UV radiation, inflammatory processes, but also in diseases such as psoriasis, atopic dermatitis, contact dermatitis, virtiligo, etc. [Portugal et al., 2007].

Photoaging is a multisystem degenerative process, involving the skin and the cutaneous support system (bones, cartilage, subcutaneous compartments), being characterized by a series of skin tissue transformations over time, following exposure to UV radiation [Han et al., 2014]. During the skin aging process, the efficiency of the

endogenous antioxidant system is diminished, and the ROS formation mainly causes DNA damage, but also intracellular lipid peroxidation, abnormal oxidation reactions of proteins, all causing cell damage, inflammation, immune suppression, oxidative stress, hormonal imbalances and premature aging.

In homeostatic conditions, the skin surface is colonized by a variety of microorganisms, such as bacteria, viruses, fungi, arthropods, etc. However, a dynamic and healthy balance between the epidermis and the microorganism's population is regulated by the antibiotic and antifungal compounds production, by dermal sebocytes, as well as by the microorganisms themselves involved [Schommer et al., 2013]. Skin tissue serves as the first line defense against microbial infections, by secreting sebum with acidic pH and fatty acids to inhibit pathogens and by possessing its own normal flora, thus preventing colonization by other pathogens.

Malignant cutaneous melanoma is the cause of over 75% of deaths associated with skin cancer and occurs most frequently among young people, being characterized by high capacity for invasion and metastasis. A significant contribution to the disease development and progression is made by both genetic factors and exposure to UV radiation and the tumor microenvironment. [Cotignola et al., 2007]. Repeated exposure of skin tissue to UV radiation influences the functioning and survival of many cell types, being the causative factor of skin cancer. Skin pigmentation is the most important photoprotective factor, due to melanin, which in addition to absorbing UV radiation, also has regulatory properties on epidermal homeostasis, elimination of free radicals to protect against oxidative damage and possibly even antimicrobial activity. [Brenner et al., 2008]

Over time, researches has shown that enriching the human body's systems with natural antioxidants can correct the imbalance of homeostasis, prevent the onset of diseases caused and / or stimulated by oxidative stress mediated by free radicals, but can also play a significant role in treating these conditions. These considerations have accelerated the search, isolation and refinement of active antioxidant principles. Plant extracts are increasingly used as phytotherapeutics and are important sources of natural antioxidants, which strengthen endogenous antioxidant defense against damage caused by reactive oxygen species (ROS) and restore optimal balance by neutralizing these reactive species [Tiwari, 2004] .

Plant bioactive compounds can be defined as plants secondary metabolites that generate pharmacological or toxicological effects in humans and animals. Plants produce various antioxidant compounds, enzymatic and non-enzymatic, to counteract the effect of ROS [Asif, 2015]. Fruits and vegetables are primary sources of natural antioxidants, containing a variety of antioxidant compounds such as vitamin C, vitamin E, carotenoids, lutein, lycopene, phenolic compounds (flavonoids, tannins, lignin's), etc. [Altemimi et al., 2017]. Secondary metabolites are produced in plants in addition to the primary biosynthetic and metabolic pathways for compounds associated with plant growth and development and are considered secondary biochemicals that are not necessary for its daily functioning. Epidemiological studies suggest that an intake of natural compounds is associated with decreased risk of cancer, cardiovascular and neurodegenerative diseases, as well as conditions caused by oxidative stress. [Asif, 2015].

Historically, natural products have been the source of the active ingredients of therapeutic agents, especially in the fields of infectious diseases and tumor therapy. Natural products play an irreplaceable role in the discovery of new drugs and new drug precursors, being an important source of new structures and having a unique role in the development of anti-tumor compounds. Today, more than half of the world's widely used anti-cancer drugs come from natural sources. Natural compounds are well characterized as having a wide variety of anti-tumor properties, for example, inducing apoptosis and autophagy and inhibiting cell proliferation.

PART II. PERSONAL CONTRIBUTIONS

INTRODUCTION

In the restoration of cellular homeostasis affected by the progressive impact of exogenous or endogenous free radicals, regulatory mechanisms are involved. This presume processes strictly directed through signal molecules, intra- and intercellular factors and interactions that can be modulated by natural compounds through their structural complexity, succeeding a multiple impact through convergent pathways.

The central idea of this research starts from highlighting some plant complexes, active in combating oxidative stress on the skin, with complementary action in related degradative mechanisms, generating high incidence, serious dermatological pathologies. These cellular processes refer to: apoptosis, proliferation and turnover rate, cytokine modulation of inflammation and correlation at the vascular endothelium level with the expression of adhesion molecules, enzyme synthesis / degradation ratio in the formation of extracellular matrix proteins, keratinocyte differentiation, tumor invasion.

For the selection of active plant complexes, plants from *Salvia officinalis, Corylus avellana, Arctium lappa, Solanum lycopersicum, Trifolium pratense, Vitis vinifera* species were studied, orienting us towards the

capitalization of polyphenolic structures (grape resveratrol, myricetin and anthocyanins from hazelnut, caffeic acid, chlorogenic acid from burdock), triterpenic structures(sage ursolic acid), glycaloalkaloid structures (tomato steroidal glycaloalkaloids), isoflavones (genistein, daidzein, formononetin, red clover biochanin), burdock polyacetylene.

Researches begins with a general screening, at the acellular level, on the antioxidant / antiradical action of some plant extracts from *Salvia officinalis* (**SvPG** - extract with polyphenols and triterpene acids); *Corylus avellana* (**Al** - polyphenylcarboxylic acid extract, flavone); *Arctium lappa* (**Br** - polyphenylcarboxylic acid extract, sesquiterpenes); *Trifolium pratense* (**ET** - phytoestrogenic extract) and active components of vegetable waste from *Solanum lycopersicum* (**Sol-T** - steroid glycoalkaloid extract) and *Vitis vinifera* (**TES** - polyphenolic extract) to establish their potential to combat the oxidative attack. The results will direct subsequent investigations to the thoroughgoing study of the antioxidant effects at the cellular level, in the enzymatic system (superoxide dismutase and catalase) and intracellular oxygenated free radicals (superoxide anion and hydrogen peroxide). On the other hand, characteristic mechanisms of skin degenerative pathologies, defining for the progression of the disease (eg inflammation, proliferation and apoptosis, extracellular protein matrix restoration, reepithelialization, malignancy and metastasis in melanoma) will be investigated, highlighting associations of active plant complexes in these processes. The combinations of plant extracts will be selected based on the antioxidant profile resulting from the complex acellular and cellular screening and the structure-biological activity relationship. (**Figure 4**)

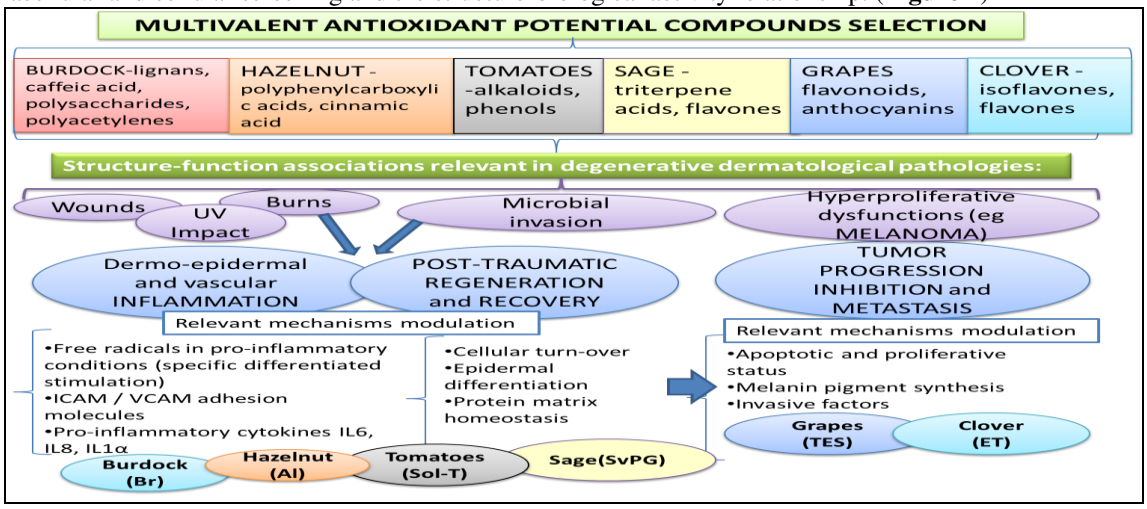


Figure 4. Highlighting representative cellular and molecular processes for degenerative dermatological pathologies, modulated by natural active compounds with multivalent antioxidant potential

The experimental models developed and applied in this study provide specific stimulation in order to mimic in vitro particular pathological condition:

TNF-α – strong pro-inflammatory stimulus, generator of non-specific inflammation - relevant in dermatological diseases with inflammatory component: dermatitis, psoriasis, wounds of different etiologies, etc.;

LPS - lipopolysaccharide, generator of inflammation associated with bacterial invasion, involved in skin mycoses and superinfection of wounds;

PMA - (phorbol acetate myristate) stimulus that generates oxidative stress associated with inflammation;

UV-A and UV-B with controlled intensity, to mimic the radiant impact on skin tissue - photosensitization, sunburn.

The results we project will contribute to highlighting interrelated cellular mechanisms in the progression of dermatological pathologies with high incidence, having as central triggering factor oxidative stress. An important aspect will be the demonstration of effects at the level of cell and signal molecule induced by biologically active complexes from native plants and vegetable waste, on the basis of which relevant therapeutic or prevention schemes can be configured.

CHAPTER 4. METHODOLOGICAL ALGORITHM OF THE EXPERIMENTAL STUDY

4.1 EXPERIMENTAL MODELS

4.1.1. Antioxidant / antiradical activity in the acellular system achieved by evaluating the active principles of *Salvia officinalis*, *Arctium lappa, Corylus avellana, Trifolium pratense* and *Vitis vinifera* species, in

terms of the ability to capture free radicals, spectrophotometric methods are applied which determine on the one hand reduction of DPPH and ABTS free radicals and on the other hand the luminol-hydrogen peroxide couple generated by luminescent emission. The obtained results represent a prediction stage and suggest the orientation towards the development and elaboration of *in vitro* experimental screening models of antioxidant parameters in relevant cellular systems at dermo-epidermal level.

4.1.2. Intracellular oxidative stress evaluation by determining the effect of phytocompounds from *Salvia officinalis, Arctium lappa, Corylus avellana, Solanum lycopersicum, Trifolium pratense* and *Vitis vinifera*, on the endogenous enzymatic oxidative system (phase I oxidative enzymes - superoxide dismutase and catalase), generators of reactive oxygen species (superoxide anion - O_2^- and hydrogen peroxide - H_2O_2), evaluated by spectrophotometric and flow cytometry techniques. To highlight the antioxidant action of the active principles tested, standardized cell lines of fibroblasts (HS27), keratinocytes (HaCaT) and vascular endothelial cells (HUVEC) are used, with mimicking conditions of nonspecific systemic inflammation (TNFα stimulation), associated with endogenous oxidative stress (PMA stimulation) and with bacterial inflammation (LPS stimulation).

4.1.3. Inflammatory status at the dermo-epidermal and vascular level evaluated by monitoring some representative parameters involved in inflammatory processes. In the damaged skin tissue, the IL1α, IL6 and TNFα cytokines are strongly expressed, and the IL8 chemokine also intervenes in the process of reepithelialization and angiogenesis, by stimulating the migration of inflammatory cells. The experimental model for determining the anti-inflammatory action of the tested plant extracts aims to determine the secretion of IL6, IL8 and IL1α pro-inflammatory cytokines, VEGF factor, angiogenesis promoter and adhesion molecules expressed on the surface of endothelial cells - ICAM and VCAM, secreted by fiber , keratinocytes and endothelial cells, on which schemes have been applied to simulate inflammatory aggressions generated either by a bacterial attack or by nonspecific systemic inflammation, accompanied by the onset of endogenous oxidative stress.

4.1.4. Photoprotective effect demonstrated by monitoring the main cellular parameters with impact in the pathogenesis of photoaging, respectively: cellular apoptosis, cell cycle sequence disturbances, intracellular oxidative stress (O_2^- and H_2O_2), inflammation (IL6, IL8, and IL1α), and angiogenesis (VEGF). Cellular mechanisms modulation under the UV radiation influence is performed at the level of normal human keratinocytes treated with extracts of *Salvia officinalis* and *Corylus avellana*.

4.1.5. Skin regenerating effect by stimulating the process of keratinocyte differentiation but also by maintaining the homeostasis of the extracellular matrix at the dermal level. In vitro keratinocyte differentiation is regulated by external calcium concentration, which induces well-defined differentiation stages in which proteins such as involucrin, transglutaminase and keratin K14 are specifically expressed, highlighted by labeling with specific antibodies and visualized by flow cytometry. The role of the active principles studied in the dermal remodeling process is determined by specific methods of monitoring the process of collagen fiber synthesis, but also by evaluating the matrix metalloproteinases (MMP9 and MMP2) enzymatic activity involved in tissue remodeling and wound healing..

4.1.6. Tumor inhibition and metastatic progression evaluation by monitoring cellular and molecular processes in the pathology of hyperproliferative disorders, in the presence of biologically active phytocompounds from red clover in association with active principles from grape waste. The mechanisms involved in the targeted melanomic progression are: melanin production, the main indicator of malignancy in skin cancers; promoters of tumor invasive character: metalloproteinases (MMP 2 and 9), soluble factors VEGF (pro-angiogenic) and IL6 (pleiotropic cytokine, modulator of intercellular signaling cascades that converge to an aggravating prognosis in malignant melanoma), proliferation and apoptosis under UV irradiation conditions.

4.3. PLANT MATERIALS AS SUPPLIERS OF ACTIVE INGREDIENTS

The active principles studied were obtained by means of a standardized extract previously developed in the Biotehnos laboratories from *Salvia officinalis, Corylus avellana, Arctium lappa, Solanum lycopersicum* species, the biocomponents combinations of *Vitis vinifera* (waste wine - marc) - and phytoestrogens from *Trifolium pratense*.

Table no.2. Tested plant extracts description

Name extract	Raw material	Composition	Preparation method
SvPG	*Salvia officinalis*	- ursolic acid– 0.144g/ 100g; - oleanolic acid– 0.057g/ 100g; - polyphenols – 0.189g/ 100g	Conditional extract in propylene glycol

Br	*Arctium lappa*	- polyphenylcarboxylic acids - 0.32g/ 100g	Conditional extract in propylene glycol
Al	*Corylus avellana*	- polyphenylcarboxylic acids – 2.32g/ 100g - flavones – 1.274g/ 100g - proanthocians - not identified	Conditional extract in propylene glycol
Sol-T	*Solanum lycopersicum*	- steroidal glycoalkaloids	DMSO soluble powder (working stock – 80 mg / ml)
TES	Grape pomace (*Vitis vinifera*)	- residue obtained from the vinification process - Aminoacids - Fatty acids (linoleic acid, oleic acid, stearic acid) = 2.8818 gr% - Polyphenols - Flavones - Anthocyanins	TES - 100mg powder / ml water (dried pomace at 45ºC, vacuum and crushed)
ET	*Trifolium pratense*	- conditioning in propylene glycol - Daidzein = 1,125 mg/100ml; - Genistein =3,98 mg/100ml; - Biochanin = 12,6 mg/100ml - Formononetin = 22,63 mg/100ml	ET – Conditional extract in propylene glycol
TES:ET	Marc (*Vitis vinifera*)		Combinations: TES:ET_A1= 9:1; TES:ET_A2= 5:1; TES:ET_A3= 3:1; TES:ET_A4= 9:2.
	Trifolium pratense		

4.4. RELEVANT STANDARDIZED CELL LINES TESTED IN EXPERIMENTAL STUDIES TO DETERMINE PRODUCT EFFECT

To demonstrate the therapeutic effect of the phytocompounds contained in the studied bioactive extracts, *in vitro* tests were performed on the following relevant standardized cell lines:

- **HaCaT (normal human keratinocyte) cell line.**
- **HS-27 (normal dermal fibroblast) cell line.**
- **HUVEC (endothelial cells) cell line.**
- **B16-F10 (murine melanoma) cell line**

CHAPTER 5. REZULTS AND DISCUSSIONS

The pathogenicity of skin diseases involves complex phenomena of a physiological, immunological, genetic, etc. nature, generated by both oxidizing agents and the cytokine network, being involved in skin disorders with varying degrees of aggression, which can even lead to carcinogenesis, lesions caused by UV radiation, inflammatory processes, as well as in conditions such as psoriasis, atopic dermatitis and contact dermatitis, virtiligo, etc. Also, chronic exposure of skin tissue to UV radiation induces a cascade of biochemical reactions with multifactorial biological responses, including the development erythema, hyperplasia, photoaging, and melanogenesis.

The presented study aims the influence of active principles such as polyphenols, triterpene acids, flavonoids, glycoalkaloids, anthocyanins, phytoestrogens, etc., on physiological and pathological processes with impact at the cellular level.

The *in vitro* study begins with the determination of the cytotoxic profile of the complexes of *Salvia officinalis, Arctium lappa, Corylus avellana, Trifolium pratense* and from the wastes of *Solanum lycopersicum* and *Vitis vinifera*, at the level of standardized cell lines of fibroblasts (HS-27), keratinocytes (HaCaT), vascular endothelial cells (HUVEC) and murine melanoma cells (B16-F10), thus establishing cytotoxicity limits.

The experimental course is aimed to evaluating the action of active principles processed from well-selected plant material on the following cellular mechanisms specific to skin tissue:

- Modulation of metabolic pathways to counteract reactive oxygen species involved in the initiation and spread of various skin disorders;
- The potential anti-inflammatory effect of biocomplexes on the skin in the conditions of mimicking inflammatory aggressions (fungal or bacterial attack, nonspecific inflammation);
- Modulation of dermo-epidermal cellular mechanisms under the influence of UV radiation, with exemplification for *Salvia officinalis* and *Corylus avellana* extracts;
- Evaluation of the skin regenerating effect through the contribution in the epidermal renewal process and in the post-traumatic regeneration in injuries such as burns, wounds, skin infections, etc.
- Study of cellular and molecular processes associated with hyperproliferative disorders (melanoma), by testing the biocomplexes from *Vitis vinifera* and *Trifolium pratense* associated, in melanoma cells both under normal conditions and after irradiation with UV radiation.

5.1. Cytotoxicity studies: cytotoxic profile of bioactive complexes from Salvia officinalis, Corylus avellana, Arctium lapa, Solanum lycopersicum, Trifolium pratense, Vitis vinifera

The limits of cytotoxicity, of the target active principles on the cell lines used in the experimental models presented in Chapter 4.4, are assessed by establishing the correlation between decreased cell viability (MTS test) and increased enzymatic activity in the culture medium (LDH test). The working protocol is described in Chapter 4.2. (Method 4.2.4.)

Evaluation of the cytotoxicity of the compounds used was performed on standardized cell lines of normal human fibroblast (HS27), human keratinocyte (HaCaT), normal endothelial cells (HUVEC), murine melanoma cells (B16-F10), depending on the targeted action at the cellular level.

The cellular inoculum (7000 cells / well) was adhered for 24 h subsequently adding the studied extracts and incubated to the culture for 48 h to evaluate the cytotoxic / cytostatic potential. Cells were treated with serial dilutions of each extract as follows: **SvPG** extract was tested over a percentage concentration range of 0.006% - 1%; **Br** and **Al** extracts were evaluated in the concentration range 0.025% - 2%; the **Sol-T** extract was diluted in the range 0.4 - 12 µg / ml; **ET** extract was tested in the range 0.007% - 0.5%; the **TES** extract was diluted in the range of 1 - 30 mg / ml. After marking and recording the absorbance, the values were mathematically processed and the ratio between the effect of the samples / the effect of the control as a function of the tested concentration was plotted.

Table no.7. Cytotoxicity threshold for biocomplexes studied on target cell lines:

Cell line / Substance	HaCaT	HUVEC	HS27	B16-F10
	Dose extract			
SvPG	0,1%	0,011%		
Al	0.167 %	0.166 %	0.100 %	
Br	2.5%	0.166%	0.5%	
Sol-T	1 µg/ml	6 µg/ml	0.1 µg/ml	
TES	5 mg/ml		5 mg/ml	10 mg/ml
ET	0.02 %		0.033 %	0,003%
TES:ET_A1			2%	2%
TES:ET_A2			2%	2%
TES:ET_A3			2%	3%
TES:ET_A4			Nontoxic	Nontoxic

The cytotoxic profile of bioactive complexes determined in cytotoxicity studies, is the starting point for subsequent tests performed on cellular systems relevant to the target mechanisms, based on which prevention and / or therapeutic schemes can be configured.

5.2. Antioxidant / antiradical activity of bioactive extracts from Salvia officinalis, Corylus avellana, Arctium lappa, Solanum lycopersicum, Trifolium pratense, Vitis vinifera studied in the acellular system

Reactive oxygen species (ROS) are continuously generated in normal cellular metabolic processes and induced by external factors. At physiological concentrations, free radicals play an essential role in a number of important biological processes such as: cellular signaling; influencing the cellular response; control of viability, migration and cell differentiation; protection of cells against pathogens and infectious agents by inactivating them [Molyneux, 2004]. A higher level of ROS than the physiological one, causes an disruption in the balance of

oxidative species - antioxidants (oxidative stress), thus inducing diseases such as atherosclerosis, cardiovascular disease, diabetes, inflammation, cell aging, skin lesions, rheumatoid arthritis and neurological diseases [Sies, 2018]. When endogenous antioxidants of an enzymatic or non-enzymatic nature are no longer able to counteract the negative effect of ROS, it is essential that the human organism receives a supply of natural antioxidants such as phenolic acids, tocopherols, flavonoids, etc. to exert its effect through various mechanisms such as inhibition of hydrogen atom release, binding of transition metal ions, radical scavenger, disintegration of peroxides [Kohen et al., 2002].

In order to highlight the antioxidant / antiradical capacity of the extracts obtained by applying extraction methods specially developed and optimized for isolate the interest compounds from the plant material as efficiently as possible, a series of experiments were performed in the acellular system aimed at their potential to eliminate reactive species from the test environment [Christmas et al., 2020] . Given the phytochemical composition of the extracts, bioactive complexes (**SvPG, ET, TES, Br** and **Al**) with high content in compounds such as polyphenols (flavonoids, polyphenolcarboxylic acids and proanthocyanins), triterpene acids, phytoestrogens were tested for antioxidant / antiradical screening. In vitro evaluation of the potential effect on oxidative stress in the acellular system was performed by applying the methods described in detail in *Chapter 4.2: The total antioxidant status evaluation by assessment the reduction of the ABTS radical (method 4.2.1.)*; *Antiradical activity determination - DPPH radical reduction method (method 4.2.2.)*; *Antioxidant activity determination by chemiluminescence using the peroxidase-luminol-H_2O_2 couple (method 4.2.3.)*; the absorbance values recorded at the test-specific wavelength being mathematically processed and presented as a graph (see **Figures 14, 15** and **16**). From their analysis it is observed that the absorbance values decrease in a dose-effect manner with a linear dependence.

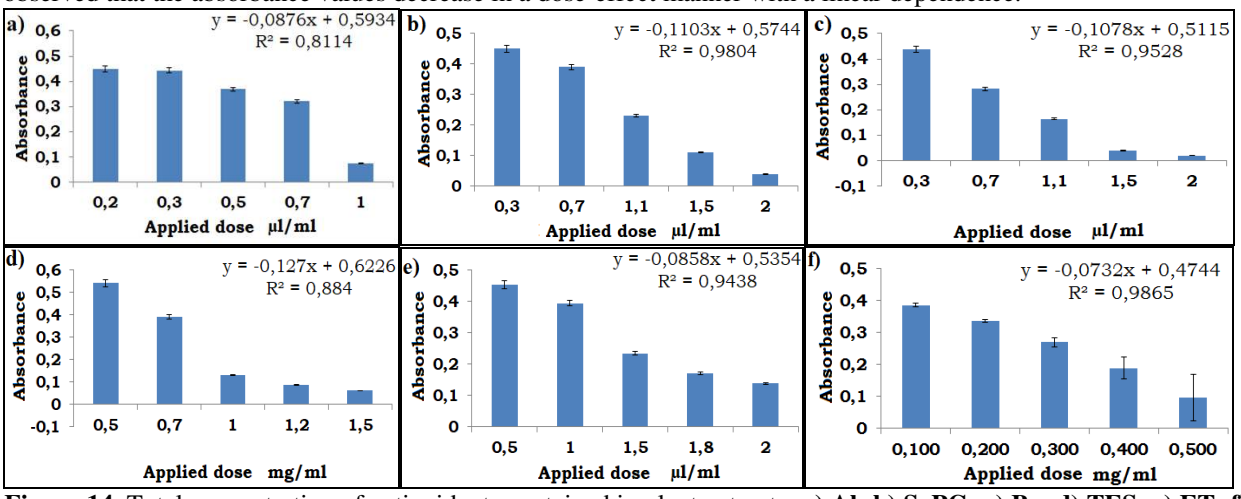


Figure 14. Total concentration of antioxidants contained in plant extracts: **a) Al; b) SvPG; c) Br; d) TES; e) ET; f) Vitamin C** evaluated on the basis of the ability to reduce the ABTS radical depending on the dose of extract applied

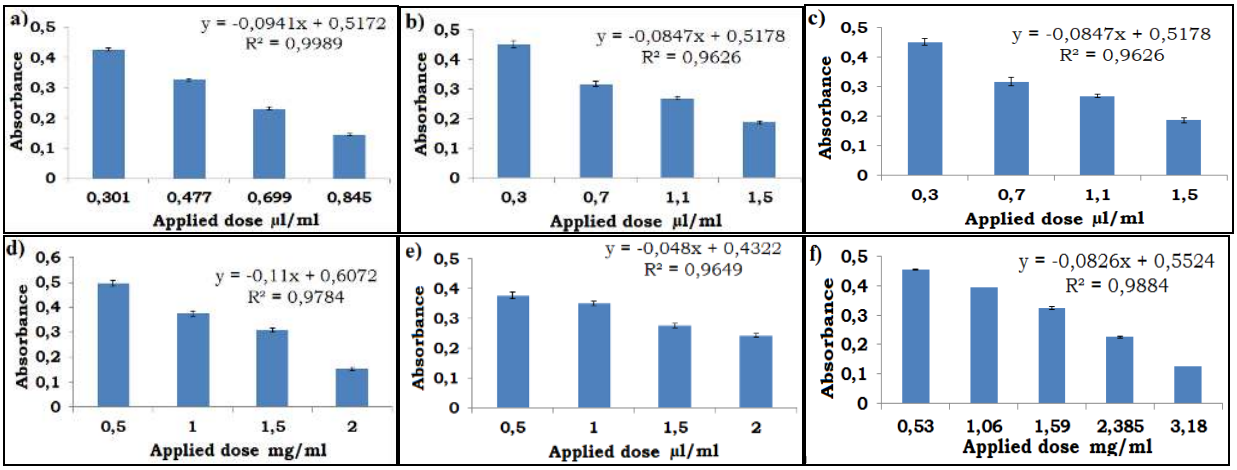
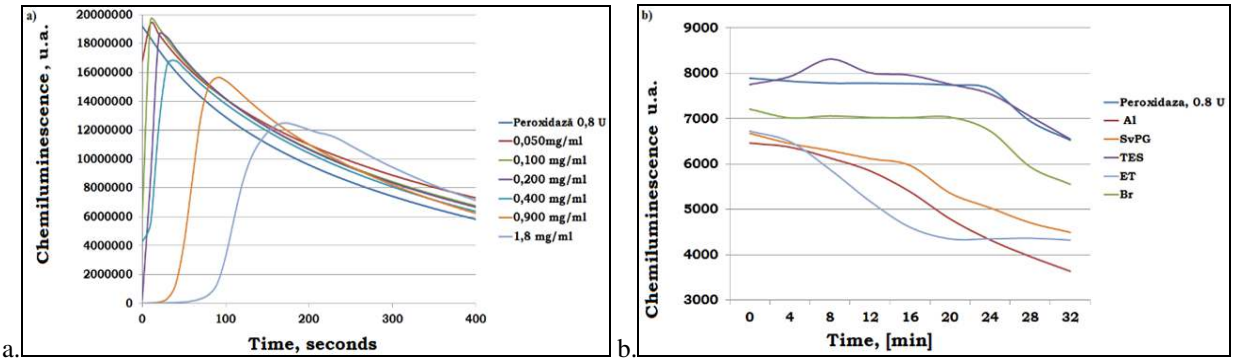


Figure 15. Antiradical capacity of extracts **a) Al; b) SvPG; c) Br; d) TES; e) ET; f) vitamin C** evaluated on the basis of the free radical DPPH depending on the applied dose



a. b.

Figure 16. Antioxidant activity evaluation through a peroxidase-luminol-H_2O_2 coupled system. a) Kinetics of the chemiluminescent signal in the presence of positive control - vitamin C depending on the applied dose; b) Extinguishing of the chemiluminescent signal in the presence of extracts (the applied dose is equivalent to the EC_{50} established in the DPPH reduction test)

The curves shown in **Figures 14-16** led to the determination of EC_{50} values (the minimum dose of extract necessary to reduce by 50% the concentration of stable free radicals generated in the system - Table no.8), based on which, following some processing advanced mathematics [Chang et all., 2010], the antioxidant action pattern is established.

Table no.8. Antioxidant activity of biocomplexes isolated from plant material

Sample		EC_{50} (µl extract)* (*) The results represent the mean ±S.D., n=3		
		ABTS	**DPPH**	**CL**
Evaluated extract:				
1.	SvPG (µl/ml)	0.47±0.0141	1.724±0.300	0.10±0.0010
2.	Al (µl/ml)	0.31±0.006	0.397±0.008	0.09±0.0005
3.	Br (µl/ml)	0.41±0.010	0.940±0.150	0.10±0.0015
4.	ET (µl/ml)	0.70±0.021	1.869±0.056	0.05±0.0015
5.	TES (mg/ml)	3.98±0.123	1.330±0.027	0.08±0.0024
Positive control:				
6.	Vitamin C (mg/ml)	0.357±0.007	1.6±0.04	0.42±0.006

The graphical representation (see **Figure 17**) of the samples (see numbering table number 8) compared to the EC_{50} value forms the map of the sample distribution according to the three applied methods, giving the possibility to mathematically determine the correlation coefficient (r) (r = 0.0897 - antiradical effect evaluated based on the reduction of DPPH, 0.1571 - reduction of the ABTS radical and 0.3445 - antioxidant effect by coupling peroxidase-luminol-hydrogen peroxide). The blue and orange lines intertwine (**Figure 17**), which indicates that the molecules with antioxidant effect contained in the studied biocomplexes are unlikely to act in several antiradical ways.

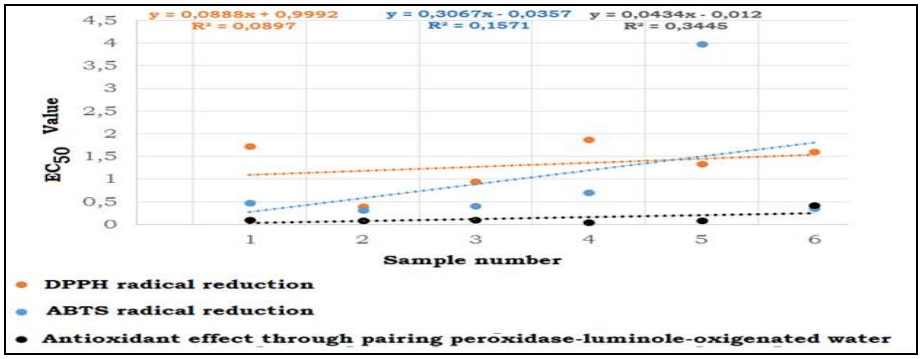
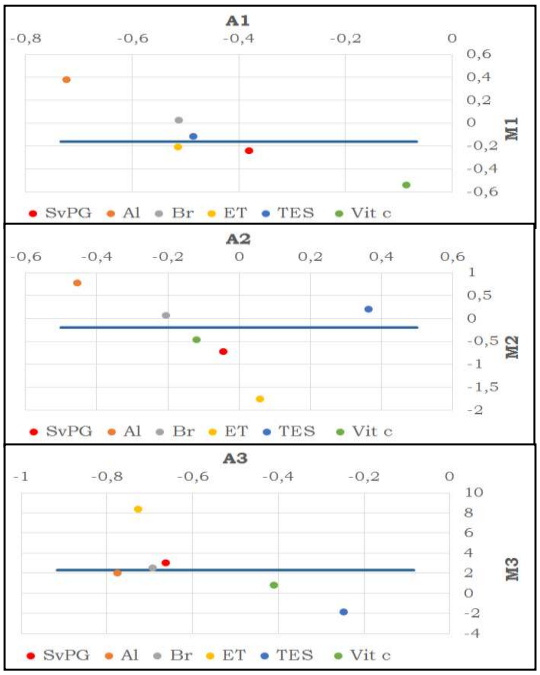


Figure 17. Graphical representation of the three methods for assessing the potential antioxidant/ antiradical.

The M-A type graphical representation was used to highlight the differences in antioxidant / antiradical action of the samples depending on the three methods applied. In an Excel file, were calculated the values for:

- M1 = log [(EC_{50} of the sample evaluated by the DPPH reduction method) / (EC_{50} of the sample evaluated by the coupled Peroxidase-luminol-hydrogen peroxide system)]
- A1 = [log (EC_{50} of the sample evaluated by the DPPH reduction method) + log (EC_{50} of the sample evaluated by the coupled Peroxidase-luminol-hydrogen peroxide system)] / 2
- M2 = log [(EC50 of the sample determined by the DPPH reduction method) / (EC50 of the sample evaluated by the ABTS reduction method)]
- A2 = [log (EC50 of the sample evaluated by the DPPH reduction method) + log (EC50 of the sample evaluated by the ABTS reduction method)] / 2
- M3 = log [(EC50 of the sample determined by the Peroxidase-luminol-hydrogen peroxide coupled system) / (EC50 of the sample evaluated by the ABTS reduction method)]
- A3 = [log (EC50 of the sample evaluated by the coupled Peroxidase-luminol-hydrogen peroxide system) + log (EC50 of the sample evaluated by the ABTS reduction method)] / 2



In the graphical representation of type M1-A1, the equivalent point for **Br** on the y-axis (M1) is located at zero and the median is very close to zero, a similar situation encountered for the representation of M2-A2 with the mention that the other points have a larger scatter and are positioned at a considerable distance from zero. In the graphical representation M3-A3 positioned very close to zero is Vit C, but the average value is far from zero.

The submission of the experimental results to this analysis process had as objective the determination of an antioxidant mechanism according to the experimental values obtained by applying the three methods, as follows:

- **Br** extract acts mainly through stable and very weak free radical reduction mechanisms on ROS,
- **ET** acts only as a scavenger for free radicals,
- **TES -** can be used both as a scavenger for free radicals and as a complex with antioxidant action through mechanisms to reduce reactive oxygen species
- **SvPG** – compared to the other complexes studied, it has a low antiradical / antioxidant potential
- **Al** – the active principles contained in this extract manifest their antioxidant potential by acting in the sense of reducing reactive oxygen species.
- **Vit C** – is a powerful antioxidant that acts mainly on the mechanisms of counteracting ROS, an aspect confirmed by the literature.

The use of Vit C as a reference substance validates the experimental model developed, optimized and applied in this extensive study.

From the analysis of the data obtained, there is an antioxidant effect in all five extracts studied, which by their ability to capture free radicals, can play a role in combating cell membrane degradation, destruction of membrane proteins or mutations in the DNA molecule, thus preventing the spread and development of many diseases (atherosclerosis, cancer, diabetes, liver disease, inflammation, skin lesions, arthritis, etc.).

These preliminary results were the basis for the development and elaboration of experimental in vitro models for antioxidant screening at the cellular level aimed to modulating the main metabolic pathways to counteract reactive oxygen species (superoxide anion and hydrogen peroxide) involved in the initiation and spread of various skin pathologies in order to demonstrate the potential antioxidant effect of plant biocomplexes.

5.3. Intracellular oxidative stress modulation by Salvia officinalis (SvPG), Corylus avellana (Al), Arctium lapa (Br), Solanum lycopersicum (Sol-T), Trifolium pratense (ET) and Vitis vinifera (TES) extracts in cellular systems with dermo-epidermal relevance

The imbalance between the appearance of reactive oxygen / nitrogen species (ROS / RNS) and the body's ability to counteract their action through antioxidant protection systems, leads to damage to important biomolecules and cells, with potential impact on the whole organism [Yoshikawa et al., 2000].

ROS are generated during normal metabolism, are an integral part of normal cellular function, and are usually very harmful due to intracellular mechanisms that counteract their effects. Antioxidants alleviate the harmful effects of ROS and can affect and reverse many of the events that contribute to skin conditions. However, an increased or prolonged action of free radicals can affect the defense mechanisms against ROS, contributing to the development of skin diseases and disorders [Trouba et al., 2002; Sies, 2015].

The antioxidant effect evaluation of the extracts on two phase I oxidative enzymes involved in reducing intracellular oxidative stress: catalase - superoxide dismutase, was performed by two spectrophotometric methods to determine the enzymatic activity of catalase (reaction of decomposition of hydrogen peroxide in water and O_2) – Method 4.2.5. and superoxide dismutase (monitoring the inhibition of the cytochrome c reduction process by the superoxide radical) - method 4.2.6. Evaluation of intracellular oxidative stress was performed by monitoring reactive oxygen species (hydrogen peroxide and superoxide anion - labeling with DCFH-DA, respectively HE) and flow cytometry analysis (method 4.2.7.-A).

In this experimental model, the aim was to evaluate the intracellular oxidative stress in the cell lines of dermal fibroblasts (HS-27), keratinocytes (HaCaT) and vascular endothelial cells (HUVEC), both in basal and stimulating conditions: bacterial inflammation-LPS (a lipopolysaccharide extracted from the bacterium Escherichia coli - 1μg / ml) and nonspecific systemic inflammation (TNF-α, 15ng / ml), accompanied by the onset of oxidative stress (PMA, 0.1μM) [Christmas et al., 2020; Christmas et al., 2017].

The enzymatic activity evaluation of intracellular enzymes: catalase (CAT) and superoxide dismutase (SOD) secreted by dermo-epidermal cells, in the presence of bioactive extracts SvPG, Br, Al, Sol-T, ET, TES.

The determination of the extract concentration introduced in the culture medium at the extension of the model in the cellular system on the HS-27 and HaCaT lines was made based on the dose range that does not affect cell viability, established in preliminary cytotoxicity studies, presented in subchapter 5.1. From the kinetic curves recorded in triplicate, the specific enzymatic activity was determined, the calculated values being presented below:

Table no.9. Catalase and superoxide dismutase enzymatic activity modulation in HS-27 cells by the tested bioactive extracts:

Test compound	HS-27 cell line					
	AE SOD (U/ml)			AE CAT - k(nmol/min/ml)		
	unstimulated	TNFα+PMA	LPS+PMA	unstimulated	TNFα+PMA	LPS+PMA
Mc	119	98	89	6,55	8,39	9,95
SvPG 0.025%	199	124	117	8,42	11,06	12,50
Br 0.1%	188	118	115	7,16	11,62	10,76
Al 0.1%	231	136	148	8,04	10,02	12,98
Sol-T 100 ng/ml	212	131	113	12,17	8,76	10,42
ET 0.2%	177	161	143	10,31	9,66	11,68
TES 4 mg/ml	197	123	178	12,53	11,80	16,43
N-Ac-Cis 2mM	159	168	123	6,19	10,67	10,59

Table no.9-b Catalase and superoxide dismutase enzymatic activity modulation in HaCaT cells by the tested bioactive extracts:

Test compound	HaCaT cell line					
	AE SOD (U/ml)			AE CAT - k(nmol/min/ml)		
	unstimulated	TNFα+PMA	LPS+PMA	unstimulated	TNFα+PMA	LPS+PMA
Mc	104	183	105	1,35	1,97	1,48
SvPG 0.025%	159	309	162	1,59	2,07	2,42
Br 0.1%	240	188	205	1,33	2,87	2,12
Al 0.1%	256	226	125	3,46	3,26	2,22
Sol-T 100 ng/ml	265	316	170	2,61	2,25	3,34
ET 0.2%	139	114	327	1,81	6,52	2,83
TES 4 mg/ml	246	226	130	4,60	3,16	4,55
N-Ac-Cis 2mM	119	186	118	1,83	2,33	3,92

Intracellular oxidative stress evaluation by monitoring reactive oxygen species (hydrogen peroxide and superoxide anion - labeling with DCFH-DA, respectively HE) and flow cytometry analysis.

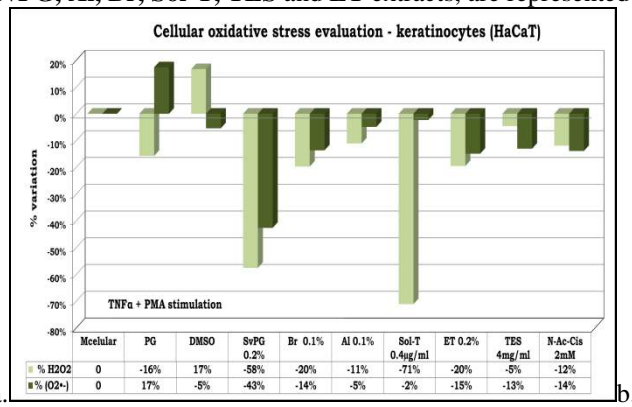
The results are presented in the graphs below, as follows: the amount of hydrogen peroxide, respectively the intracellular superoxide anion correspond to the variation of the fluorescence channel averages in the 2 coordinates: FITC - A mean - for hydrogen peroxide and PE-A mean - for the superoxide anion. In order to highlight the antioxidant action of phytocompounds, the tests performed were carrying out under the following stimulation conditions:

- Non-specific systemic inflammation (TNF-α), accompanied by the onset of oxidative stress (PMA) - TNFα 15ng / ml + PMA 0.1μM, 24 hours of stimulation.
- Bacterial inflammation: LPS, a lipopolysaccharide extracted from the bacterium Escherichia coli - 1μg / ml 18 hours of stimulation, associated with an endogenous oxidative stimulus (PMA) under the concomitant and potentially preventive action of the active substances to be tested [Christmas et al., 2020; Christmas et al., 2017].

HaCaT cell line - normal human keratinocyte

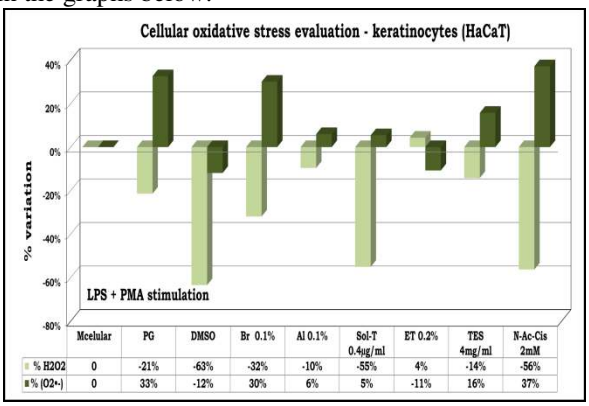
The percentage changes of reactive species generated intracellular on human keratinocyte in the presence of **SvPG, Al, Br, Sol-T, TES** and **ET** extracts, are represented in the graphs below:

a.



	Mcelular	PG	DMSO	SvPG 0.2%	Br 0.1%	Al 0.1%	Sol-T 0.4μg/ml	ET 0.2%	TES 4mg/ml	N-Ac-Cis 2mM
% H2O2	0	-16%	17%	-58%	-20%	-11%	-71%	-20%	-5%	-12%
% (O2•-)	0	17%	-5%	-43%	-14%	-5%	-2%	-15%	-13%	-14%

b.



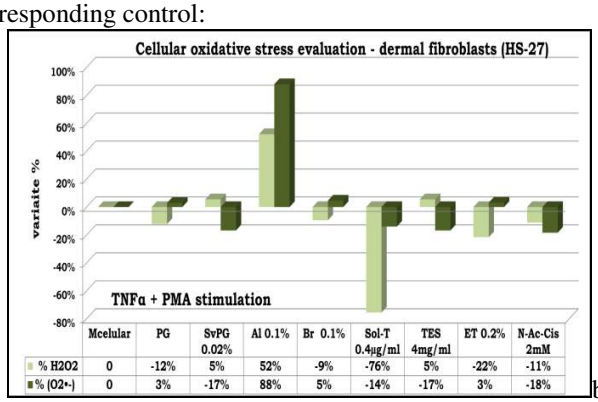
	Mcelular	PG	DMSO	Br 0.1%	Al 0.1%	Sol-T 0.4μg/ml	ET 0.2%	TES 4mg/ml	N-Ac-Cis 2mM
% H2O2	0	-21%	-63%	-32%	-10%	-55%	4%	-14%	-56%
% (O2•-)	0	33%	-12%	30%	6%	5%	-11%	16%	37%

Figure 19. Highlighting the action of phytocompounds on cellular oxidative stress by flow cytometry on HaCaT cell line: TNFα + PMA stimulation (a.); LPS + PMA stimulation (b.); N-Acetyl-cysteine were used as a positive control

HS-27 cell line - human fibroblast

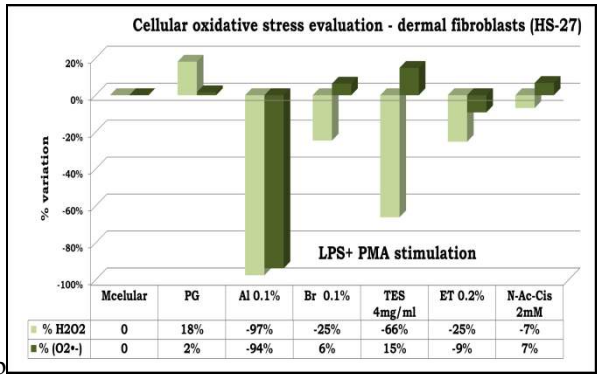
The effect of the **SvPG, Sol-T, Al, Br, ET** and **TES** bioactive extracts, on the reactive cellular oxygen species, on the HS-27 cell line, is represented in the graphs below, as a percentage variation compared to the corresponding control:

a.



	Mcelular	PG	SvPG 0.02%	Al 0.1%	Br 0.1%	Sol-T 0.4μg/ml	TES 4mg/ml	ET 0.2%	N-Ac-Cis 2mM
% H2O2	0	-12%	5%	52%	-9%	-76%	5%	-22%	-11%
% (O2•-)	0	3%	-17%	88%	5%	-14%	-17%	3%	-18%

b



	Mcelular	PG	Al 0.1%	Br 0.1%	TES 4mg/ml	ET 0.2%	N-Ac-Cis 2mM
% H2O2	0	18%	-97%	-25%	-66%	-25%	-7%
% (O2•-)	0	2%	-94%	6%	15%	-9%	7%

Figure 20. Highlighting the action of phytocompounds on cellular oxidative stress by flow cytometry at the HS-27 cell line: TNFα + PMA stimulation (a.); LPS and PMA stimulation (b.); N-Acetyl-cysteine was used as a positive control

HUVEC cell line - vascular endothelium

The effect of the **SvPG** and **Sol-T** bioactive extracts on the reactive cellular oxygen species, on the HUVEC cell line is represented in the graph below as a percentage variation compared to the corresponding control:

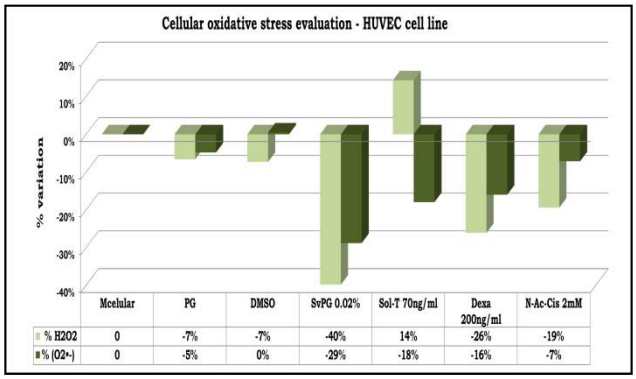


Figure 21. Highlighting the action of phytocompounds on cellular oxidative stress in endothelial cells, stimulated with TNFα + PMA by flow cytometry. Dexamethasone and N-acetyl cysteine were used as positive controls

SvPG sage extract inhibits the production of reactive oxygen species in endothelial cells stimulated with TNFα and PMA, the effect being comparable to that of the positive control tested under the same experimental conditions. The glycoalkaloid extract **Sol-T**, indicates a reducing effect on the superoxide anion under both stimulation conditions, observing a decrease of the superoxide anion concentration by 18% in the cells treated with **Sol-T** 70ng / ml and stimulated with TNFα and PMA. Under the same conditions, **Sol-T** extract generates an accumulation of hydrogen peroxide in the system, which can result in a change in vascular reactivity and can lead to toxicity and alterations in homeostasis at the vascular level.

5.4. Pro-inflammatory conditions simulation in skin tissue to assess the impact of Solanum lycopersicum, Salvia officinalis and Arctium lappa plant complexes in dermatological pathologies

A. Dermo-epidermal inflammation

Inflammation and oxidative stress are interdependent processes that can occur simultaneously or successively in the pathogenesis of many chronic diseases. Although the identification and treatment of primary dysfunctions are clinically important, the pathological status is difficult to combat due to the fact that inflammation and oxidative stress act in concert, potentiating each other to induce progressive damage. Thus, only antioxidant therapy is unlikely to prevent various pathologies caused by oxidative stress with inflammatory component such as skin lesions (chronic wounds of various etiologies, psoriasis, and acne), dermatitis or cardiovascular and diabetic complications, neurodegenerative diseases, cancer. There are, therefore, a series of premises that require research in the sense of capitalizing on natural resources with clear therapeutic potential.

Particular attention should be granted to the selection of antioxidants and the dose to achieve the desired effect. On the other hand, in the compounds testing, it is necessary to quantify the redox and inflammatory status for the correct interpretation of the results [Biswas, 2016]. If the number of activated cells is high and the inflammation manifests itself for too long, serious dysfunctions can result. There is some evidence of the anti-inflammatory role played by RONS (oxygen, nitrogen and sulfur free radicals) released by phagocytes, which may involve contributing to chemotaxis and repairing affected tissue, as well as modulating the immune response [Bickers et al., 2003].

It is known that a number of cytokines and growth factors are involved in the wound healing process which includes three general stages: the inflammatory stage consisting in extravasation of blood components producing platelet aggregation, blood clotting and migration of inflammatory cells to the site of injury; the proliferative stage involving the migration and proliferation of keratinocytes, fibroblasts and endothelial cells, leading to reepithelialization and granular tissue formation and the longer-term remodeling stage [Hübner et al., 1996].

IL-1α, IL-6 and TNFα are representative cytokines strongly expressed in inflammatory processes in damaged skin tissue. Chemokine's (IL-8) also play an active role in the wound healing process, by stimulating the migration of inflammatory cells, thus contributing to the regulation of reepithelialization, tissue remodeling and angiogenesis. During the wound healing process, angiogenesis plays a key role in the formation of new granular tissue in the proliferative phase, being evaluated by monitoring the expression of proangiogenic growth factor VEGF (vascular endothelial growth factor) [Toshikazu et al., 2010, Goodman et al. ., 2009].

The tests were performed in two experimental models, characteristic of the dermo-epidermal tissue, simulating inflammatory aggressions that may occur under fungal or bacterial attack conditions, respectively inflammation of undetermined etiology. Thus, the inflammation correlated with the cellular oxidative stress was mimicked by 2 types of stimulation:

- Bacterial inflammation: LPS, a lipopolysaccharide extracted from the bacterium Escherichia coli accompanied by the onset of oxidative stress (PMA) - 18 hours of stimulation
- Nonspecific systemic inflammation (TNF-α), accompanied by oxidative stress (PMA) - TNFα 15ng / ml + PMA 0.1μM, 24 hours of stimulation.

Inflammatory status was assessed by determining the secretion of pro-inflammatory cytokines (IL6, IL8, IL1α) on both cell types (keratinocytes and fibroblasts), and VEGF factor, promoter of angiogenesis (method 4.2.7.-B) [Christmas et al. , 2016; Christmas et al., 2017; Christmas et al., 2016].

Pro-inflammatory cytokines IL6, IL8, IL1α and proangiogenic factor VEGF evaluation under nonspecific stimulation conditions - HaCaT cell line

Knowing on the one hand the interdependence of inflammatory and oxidative mechanisms at the cellular level and the phytochemical composition of the extracts, and on the other hand taking into account the research results of the previous stage in which it was demonstrated by an extensive experimental study rich in compounds such as glycoalkaloids, triterpene acids, phytoestrogens and polyphenols, an experimental model for evaluating the anti-inflammatory effect was developed with exemplification for **Sol-T, SvPG, Al, Br, ET** and **TES** extracts. From the mentioned biocomplexes, for the evaluation of the anti-inflammatory effect, on the standardized cell line of keratinocyte type, the **Sol-T** and **SvPG** extracts were selected because in addition to the theoretical support based on literature data [Saba et al., 2017; Friedman, 2013; Siddique et al., 2019; Baricevic et al., 2001] we also have the experimental support highlighted in the antioxidant experimental model (see subchapters 5.2. And 5.3.).

Cells were treated with **Sol-T** extract and stimulated with both TNFα - 40ng / ml (mimics nonspecific systemic inflammation) and LPS - 1μg / ml (bacterial stimulus). Three different concentrations of the extract were tested, respectively 20ng / ml, 50 ng / ml, 100 ng / ml, compared to a positive control - dexamethasone 200ng / ml (known anti-inflammatory). The results are shown in the graphs below as a percentage change from the corresponding control:

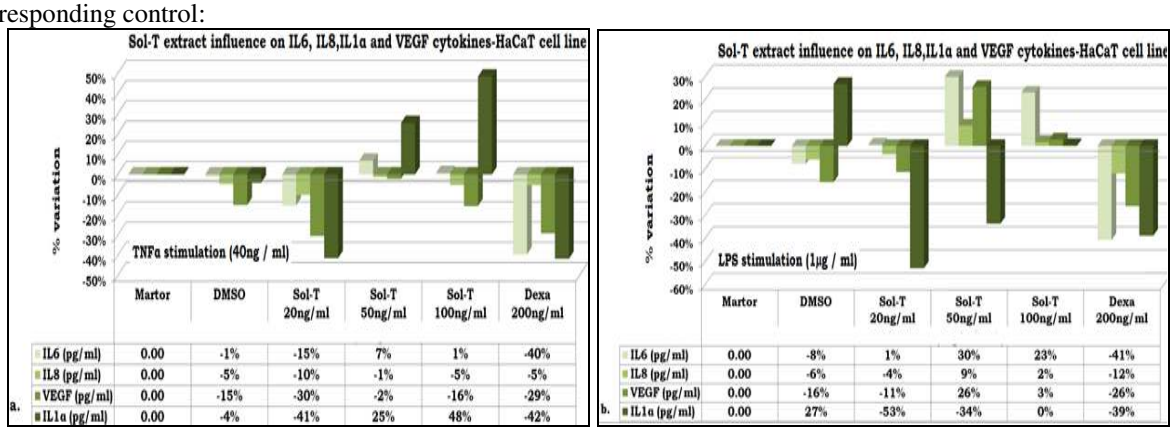


Figure 22. Percentage variation of pro-inflammatory cytokines in the presence of **Sol-T** extract on the HaCaT cell line: a. TNFα stimulation (40ng / ml); b. LPS stimulation (1μg / ml)

SvPG extract was tested for anti-inflammatory effect in human keratinocytes (HaCaT) in the presence of TNFα and PMA stimuli, and the results are plotted as a percentage change from the corresponding control as follows:

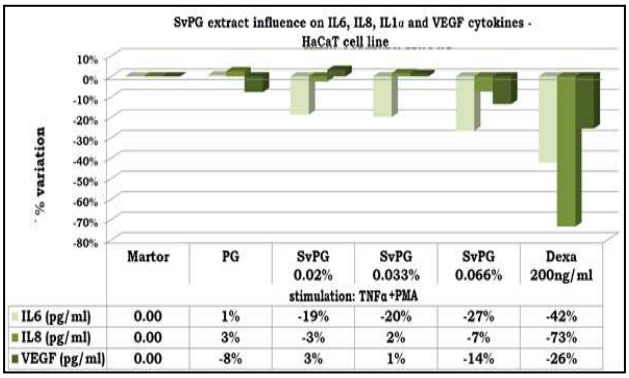


Figure 23. Percentage variation of pro-inflammatory cytokines in the presence of **SvPG** extract in keratinocytes (HaCaT cell line)

Pro-inflammatory cytokines IL6, IL8 evaluation under LPS and PMA stimulation conditions - HS-27 cell line

In order to demonstrate the anti-inflammatory effect of the active principles of **Al, Br, ET** and **TES**, at the level of the dermis, in vitro experimental models were developed, optimized and applied aimed at modulating the expression of proinflammatory cytokines (IL6, IL8) in mimicking dermal inflammation. bacterial accompanied by nonspecific prooxidant conditions.

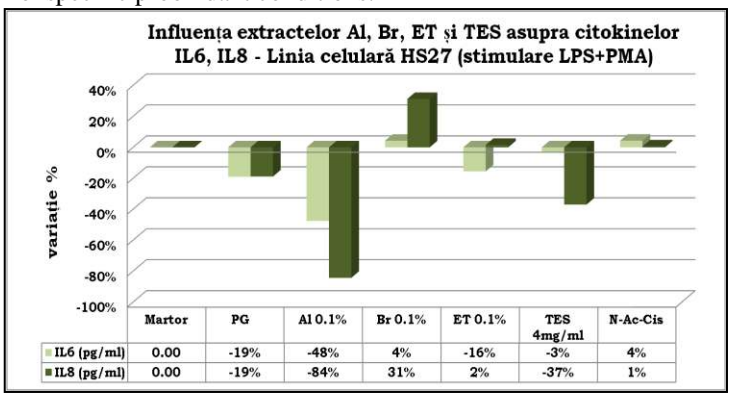


Figure 24. Percentage variation of pro-inflammatory cytokines in the presence of **Al, Br, ET** and **TES** extracts in fibroblasts (HS-27 cell line)

The glycoalkaloid extract **Sol-T** inhibits the extracellular release of cytokines IL6, IL8, IL1α, demonstrating anti-inflammatory and anti-irritant effect by blocking the signaling pathways coordinated by these cytokines. Decreasing the pro-angiogenic factor VEGF released by keratinocytes under TNFα stimulation and increasing it under the action of bacterial LPS recommends this compound in dermatological conditions without active lesions (eg dermatitis, skin irritations, etc.), respectively healing of superinfected wounds (induces microvascular formation). **SvPG** sage extract stops the extracellular signaling generated by the cytokine IL6, relevant even in severe dermatological diseases such as psoriasis. In dermal fibroblasts, under the action of a bacterial stimulus associated with an oxidative stress-generating stimulus, the **Al** extract acts on proinflammatory cytokines, in the sense of their inhibition, demonstrating a pronounced anti-inflammatory effect.

B. Vascular inflammation, importance in regeneration and post-traumatic recovery processes

Dermal endothelial cells are involved in wound healing, inflammation, tumor angiogenesis, and are predominantly of microvascular origin, being of distinct origin and functionality compared to large vessel endothelial cells, used for in vitro vascular research. The growth and turnover of endothelial cells in the skin is fundamental not only in normal development, but also in wound healing, hair follicle cycle, tumor cell metastasis, and various stages of skin pathology. In vitro endothelial cells take on a slightly elongated epitheloid shape. In recent studies, in vitro culture of primary endothelial cells is achieved by incorporating a mitogenic growth factor (VEGF) that functions as a survival factor for these cells through overexpression of Bcl-2. There are cellular components, in vitro and in vivo those synthesizes and release VEGF. [Bao et al., 2009]

One of the fundamental mechanisms of inflammation progression is the deregulation of leukocyte extravasation along the vascular endothelium and infiltration into adjacent tissue. [Swerlick et al., 1993; Peschen et al., 1999] Infiltrated leukocytes secrete a high level of inflammatory mediators, perpetuating the inflammatory response, resulting in degradation of inflamed tissue. For tissue invasion by inflammatory cells, their transmigration along the microvascular endothelium is crucial, a process mediated by adhesion molecules expressed on the surface of CAM (class cell adhesion molecules) and their corresponding leukocyte receptors. The literature data show a differentiated role of ICAM and VCAM adhesion molecules, ICAM-1 inhibition being beneficial for dermal complications, while therapies targeting VCAM-1 fight brain diseases. [Norman et al., 2008]

There are several aspects of the physiology of dermal vasculature and its involvement in the homeostasis of skin tissue. Among these we list:

- Vascular inflammation, characterized by adhesion between lymphocyte and endothelium triggered by the expression of adhesion molecules - markers of inflammation and secretion of pro-inflammatory cytokines.
- Involvement of certain cytokines, essential in endothelial activation, healing of injuries caused by UV radiation or skin wounds. For example, GM-CSF and IL1α and β induce endothelial cell proliferation, and IFN– γ and TNFα activate the endothelium through HLA-DR and ICAM-1 expression.
- The phenomenon of angiogenesis and cell proliferation

Skin anti-inflammatory agents act by inhibiting the expression of TNFα and VCAM-1, a reversible inhibition. Similarly, VCAM-1 inhibition occurs in TNFα- and IL1α-stimulated endothelial cells. Retinoids inhibit

endothelial cell proliferation, and HLA-DR effectors induce ICAM-1 expression through IFN γ, TNFα and IL1α. The anti-inflammatory effect of retinoids can also be explained by the inhibition of VCAM-1 gene expression and T cell binding to cytokine-treated endothelial cells.

The experimental systems used consisted in differentiated stimulation of endothelial cells with LPS, lipopolysaccharide that mimics bacterial infection, respectively with TNFα - nonspecific stimulus for systemic inflammation. LPS is a major component of gram-negative bacteria that acts as an endotoxin in the animal body, inducing a strong immune response and producing the secretion of pro-inflammatory cytokines. TNFα is part of the group of cytokines that stimulate the reaction of the acute phase inflammatory response.

Vascular inflammation was assessed by determination of ICAM-1 and VCAM-1 adhesion molecules by labeling with fluorescent antibodies and acquisition of flow cytometry (method 4.2.7.-B).

The relevant parameters for the physiology of the skin tissue that we will follow are:

- Stimulation with LPS 1μg / ml, bacterial stimulus, followed by analysis of ICAM expression (characteristic of dermal microvascularization), inflammatory cytokines IL6 and IL8.
- Stimulation with TNFα 20 ng / ml, nonspecific systemic stimulus, followed by analysis of VCAM expression (characteristic of large blood vessels), inflammatory cytokines IL6 and IL8.

In the following we will present the results obtained for the main types of effects pursued.

Anti-inflammatory effect in endothelial cells evaluation, by monitoring the expression of proinflammatory cytokines IL6, IL8 and adhesion molecules ICAM, VCAM, under conditions of non-specific oxidative stimulation with TNFα but also with mimicking bacterial infection with LPS - **Sol-T, SvPG** and **Br** extract.

Relevant physiological mechanism		**Vascular inflammation**			
		IL-6	**IL-8**	**ICAM-1**	**VCAM-1**
Salvia officinalis	**SvPG**	**HUVEC:** TNFα+PMA: ↓**23%** LPS: ↓**7%**	**HUVEC:** TNFα+PMA: ↓**40%** LPS**:** ↓**4%**	**HUVEC:** TNFα +PMA: ↓**12%**	**HUVEC:** TNFα +PMA: ↓**24%**
Arctium lappa	**Br**	**HUVEC:** TNFα: ↓**26%** LPS: ↓**30%**	**HUVEC:** TNFα: ↓**49%** LPS: ↓**51%**	**HUVEC:** TNFα: ↓**6%** LPS: ↓**14%**	**HUVEC**: TNFα: ↓**1%** **LPS:** ↓**7%**
Solanum lycopersicum	**Sol-T**	**HUVEC:** TNFα: ↓**18%** LPS: ↓**7%**	**HUVEC:** TNFα: ↓**23%** LPS**:** ↓**4%**	**HUVEC:** TNFα: ↑**1%** LPS: ↓**19%**	**HUVEC:** TNFα: ↓**39%** LPS: ↑**28%**

To evaluate the effect of plant extracts, **SvPG, Sol-T** and **Br** on cellular inflammatory status, vascular endothelial cells (HUVEC cell line) were stimulated pro-inflammatory with TNFα and bacterial with LPS. Among the extracts tested, **Sol-T** has a significant effect in conditions of nonspecific inflammation induced by TNFα, it acts by reducing the extracellular expression of cytokines IL6 and IL8, thus manifesting an anti-inflammatory effect at the endothelial cell level. Under the same stimulation conditions, all doses tested by **Sol-T** reduce the expression of VCAM (endothelium-monocyte adhesion molecule specific to large vessels), as well as the ICAM expression characteristic of microvascularization. The bacterial attack at the endothelial level (stimulation with LPS) is counteracted by the glycoalkaloid extract **Sol-T** which has a reducing effect on the expression of ICAM, a characteristic parameter of small blood vessels.

5.5. Impact of Salvia officinalis and Corylus avellana plant extracts on the keratinocyte - UV radiation interactions

Photo-oxidative mechanisms dependent on reactive oxygen species produced by solar radiation are the main causes of skin photo-aging and photo-carcinogenesis. Research in the field of photobiology focuses preferentially on the harmful effects of UVB and UVC radiation involved in carcinogenesis, but in recent years there has been a need for studies on the role of UVA and visibly close radiation in photodegradation and photoaging [Stojiljković et al., 2014].

UV radiation leads to upregulation of VEGF (vascular endothelial growth factor), an active pro-angiogenic factor, via the MAPK / ERK kinase-ERK1 / 2 signaling pathway (extracellular signal-regulated kinase 1/2), which leads to in vivo as the blood vessels, vascular density, and area of dermal tissue occupied by the micro-vasculature

increase in size [Debacq-Chainiaux et al., 2012]. Also, UV-A and UV-B radiation upregulates VEGF in immortalized keratinocytes (HaCaT cell line), acting indirectly on the synthesis and release of IL-1, TNF-α, and other pro-inflammatory cytokines. [Kosmadaki et al., 2003]. VEGF overexpression leads to increased vascularity of the skin, to the appearance of fragile and sinuous dermal microvasculature. Thus, VEGF secreted by keratinocytes passes through the epidermo-dermal basement membrane reaching the microvasculature of the dermis. The normal epidermis expresses a low level of VEGF, while pathological disorders such as psoriasis, contact dermatitis or wound healing overregulate the expression of VEGF in keratinocytes.

Thus, in the studies on the pathogenesis of photoaging, the following cellular parameters are important to analyze: cellular apoptosis; cell cycle sequence disturbances, oxidative stress (hydrogen peroxide released intracellularly and superoxide anion); inflammation (pro-inflammatory cytokines IL6 and IL8; IL1α); angiogenesis (VEGF factor) [Detmar, 1994]. Flow cytometry offers the possibility of simultaneous detection of hydrogen peroxide and superoxide anion released intracellularly by double fluorescent labeling with DCFH-DA (emits in FITC-A coordinates and marks hydrogen peroxide) and HE (emits in PE-A coordinates and marks the superoxide anion) .

Irradiation was performed under reproducible conditions with controlled irradiation equipment: Bio-Sun system (manufacturer Vilber-Lourmet).

In this experimental model, the aim was to study the cellular mechanisms involved in degenerative diseases of the skin tissue, having as cellular material the keratinocyte culture (HaCaT), by the following methods (described in the special chapter): evaluation of the cellular apoptosis process (method 4.2. 7.-D), in vitro testing of proliferative capacity by highlighting successive cell generations by fluorescent labeling with CFSE (method 4.2.7.-E), cell cycle sequence analysis (method 4.2.7.-F), simultaneous identification of radicals intracellular oxygenates and assessment of inflammatory status (methods 4.2.7.-A and -B).

Relevant physiological mechanism	**UV irradiation**			
	Cell apoptosis	**Cell cycle**	**Intracellular oxidative stress**	**Inflammation**
SvPG			UV A: ↓ **$O_2^{\bullet-}$**; ↓**H_2O_2** UV B: ↓ **$O_2^{\bullet-}$**; ↓**H_2O_2**	UV A: ↓**IL8** UV B: ↓**IL8**
Al	protects against apoptosis	stop cc	UV A: ↓ **$O_2^{\bullet-}$**; ↓**H_2O_2** UV B: ↓ **$O_2^{\bullet-}$**; ↓**H_2O_2**	UV A: ↓**IL6**; ↓**IL8**; ↓**IL1-α; ↓VEGF** UV B: ↓**IL6**; ↓**IL8**; ↓**IL1-α; ↓VEGF**

Exposure to UV radiation can induce genotoxic effects, which can contribute not only to the photoaging process, but also to carcinogenesis [El-Mahdy et al., 2007]. The active principles extracted from sage (**SvPG**), act by preventing the progression of early apoptosis in cells irradiated with UV-A or UV-B. **SvPG** sage extract is particularly active in reducing oxygenated free radicals, hydrogen peroxide and superoxide anion, inducing their intracellular decrease by up to 60% in the case of hydrogen peroxide generated by UV-A irradiation, and by 32% of the superoxide anion generated. of UV-B.

Al extract has the effect of intracellular reduction of reactive oxygen species especially in conditions of UV-B irradiation, without changing the impact of UV-A at this level. **Al** hazelnut extract does not act on the apoptotic process of irradiated keratinocytes, instead stops the progression in the cell cycle, both in the case of UV-A and UV-B, which means an increase in cell degradation under these specific conditions and not a protection of normal cells in the sense of preventing malignancy and stopping aberrant proliferation. **Al** extract reduces the inflammatory phenomenon and also has an antiangiogenic action in the case of UV-A radiation.

5.6. The Salvia officinalis, Solanum lycopersicum, Corylus avellana, Arctium lappa, Trifolium pratense and Vitis vinifera plant extracts action evaluation on skin regeneration

A. Epidermal regeneration by stimulating the process of keratinocyte differentiation

The HaCaT cell line is immortalized spontaneously and is the main study model in skin biology. Under typical culture conditions, the cells have a partially or completely differentiated phenotype due to the high calcium content in the culture medium and in the fetal bovine serum. Under culture conditions at low calcium concentrations, the cells undergo a reversible transformation to the basal phenotype, from which, under conditions controlled by progressive calcium concentrations, well-defined differentiation stages corresponding to the basal, spinous, granular or corneum layer can be induced.

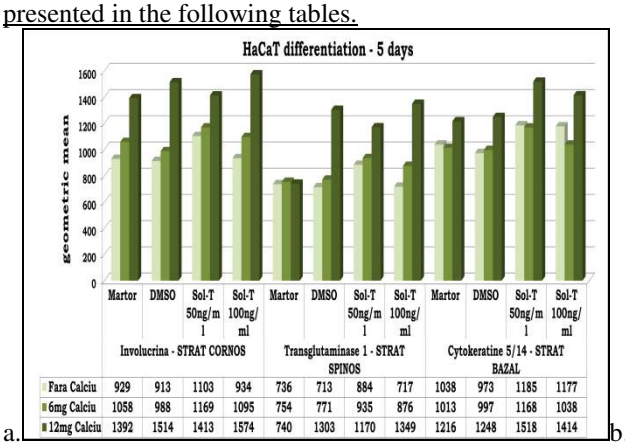
The determination of these stages of differentiation is done by highlighting certain proteins expressed specifically, as follows: Involucrin (corneum and granular layer), Transglutaminase (spinous layer), keratin K14

(basal layer). External calcium concentration regulates the differentiation of keratinocytes in vitro. Keratinocytes grown in medium with 1.2mM and 2.4mM calcium ions synthesize involucrin and transglutaminase before being confluent, being able at the confluence to form the cornified shell.

The cells were treated with the substances of interest for 5 and 7 days, respectively. Highlighting of the three membrane proteins - differentiation markers was performed by labeling with specific antibodies (Antibody for cytokeratin 5/14 conjugated with PE; Primary antibody for involucrin coupled with conjugated secondary antibody FITC; Primary antibody for transglutaminase-1 coupled with conjugated secondary antibody PE) and visualization by flow cytometry (method 4.2.7.-G) [Craciun et al., 2019].

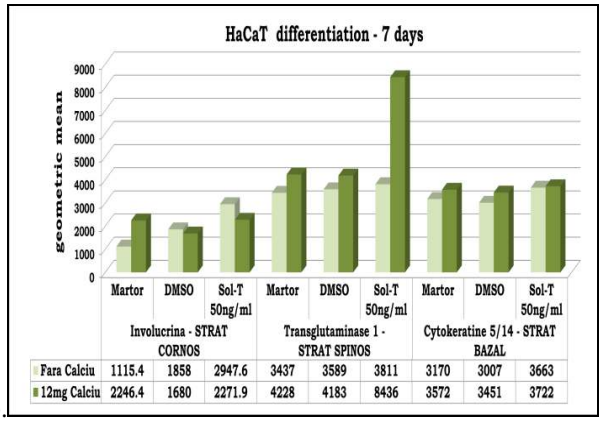
The results regarding the highlighting of the differentiation markers in the presence of **Sol-T** extract are presented in the following tables.

a.



	Involucrina - STRAT CORNOS				Transglutaminase 1 - STRAT SPINOS				Cytokeratine 5/14 - STRAT BAZAL			
	Martor	DMSO	Sol-T 50ng/ml	Sol-T 100ng/ml	Martor	DMSO	Sol-T 50ng/ml	Sol-T 100ng/ml	Martor	DMSO	Sol-T 50ng/ml	Sol-T 100ng/ml
Fara Calciu	929	913	1103	934	736	713	884	717	1038	973	1185	1177
6mg Calciu	1058	988	1169	1095	754	771	935	876	1013	997	1168	1038
12mg Calciu	1392	1514	1413	1574	740	1303	1170	1349	1216	1248	1518	1414

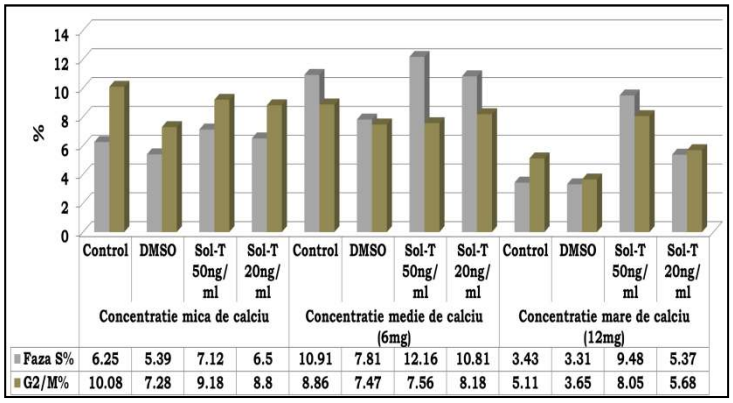
b.



	Involucrina - STRAT CORNOS			Transglutaminase 1 - STRAT SPINOS			Cytokeratine 5/14 - STRAT BAZAL		
	Martor	DMSO	Sol-T 50ng/ml	Martor	DMSO	Sol-T 50ng/ml	Martor	DMSO	Sol-T 50ng/ml
Fara Calciu	1115.4	1858	2947.6	3437	3589	3811	3170	3007	3663
12mg Calciu	2246.4	1680	2271.9	4228	4183	8436	3572	3451	3722

Figure 32. Highlighting the effect of **Sol-T** extract on the keratinocyte differentiation process after 5 (a.) And 7 (b.) Days of treatment

Proliferative status induced by **Sol-T** extract under cultivation conditions in differentiating media (with different calcium concentrations):



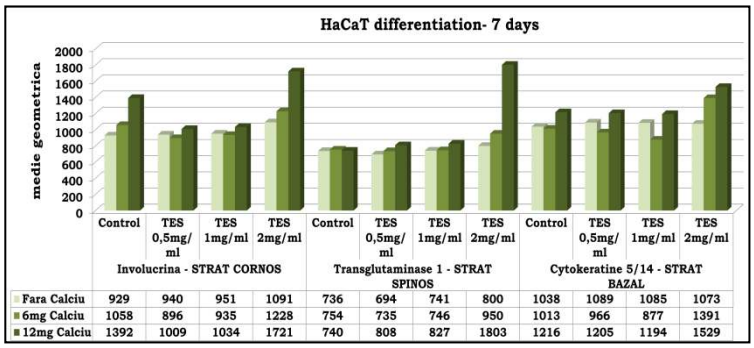
	Concentratie mica de calciu				Concentratie medie de calciu (6mg)				Concentratie mare de calciu (12mg)			
	Control	DMSO	Sol-T 50ng/ml	Sol-T 20ng/ml	Control	DMSO	Sol-T 50ng/ml	Sol-T 20ng/ml	Control	DMSO	Sol-T 50ng/ml	Sol-T 20ng/ml
Faza S%	6.25	5.39	7.12	6.5	10.91	7.81	12.16	10.81	3.43	3.31	9.48	5.37
G2/M%	10.08	7.28	9.18	8.8	8.86	7.47	7.56	8.18	5.11	3.65	8.05	5.68

Figure 33. Sequence of the cell cycle under conditions of variable keratinocyte differentiation

The **Sol-T** extract mainly induces overexpression of transglutaminase-1, hence the progression of the spinous layer. Bioactive compounds stimulate the turnover of keratinocytes both in undifferentiated conditions (calcium-poor environment) and in conditions that induce differentiation (12mg calcium), an effect that supports the process of epidermal regeneration.

Testing of bioactive compounds from **TES** complex was performed on both mechanisms.

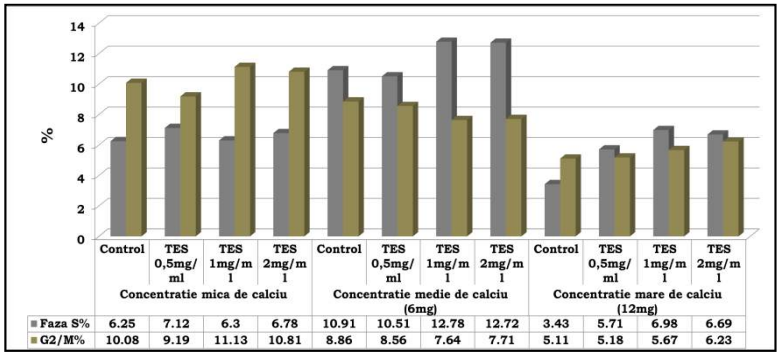
The cells are treated with the bioactive grape extract (**TES**) after 24 hours from accession and the differentiation process is analyzed after 7 days of cultivation, changing the environment every 3 days. The results are shown in the graphs below:



	Control	TES 0,5mg/ml	TES 1mg/ml	TES 2mg/ml	Control	TES 0,5mg/ml	TES 1mg/ml	TES 2mg/ml	Control	TES 0,5mg/ml	TES 1mg/ml	TES 2mg/ml
	Involucrina - STRAT CORNOS				Transglutaminase 1 - STRAT SPINOS				Cytokeratine 5/14 - STRAT BAZAL			
Fara Calciu	929	940	951	1091	736	694	741	800	1038	1089	1085	1073
6mg Calciu	1058	896	935	1228	754	735	746	950	1013	966	877	1391
12mg Calciu	1392	1009	1034	1721	740	808	827	1803	1216	1205	1194	1529

Figure 34. Expression of the three epidermal regeneration markers (Involucrin - corneum and granular layer; transglutaminase - spinous layer; cytokeratin - basal layer) under conditions of variable keratinocyte differentiation

The sequence of the cell cycle under conditions of variable keratinocyte differentiation is shown in the graphs below:

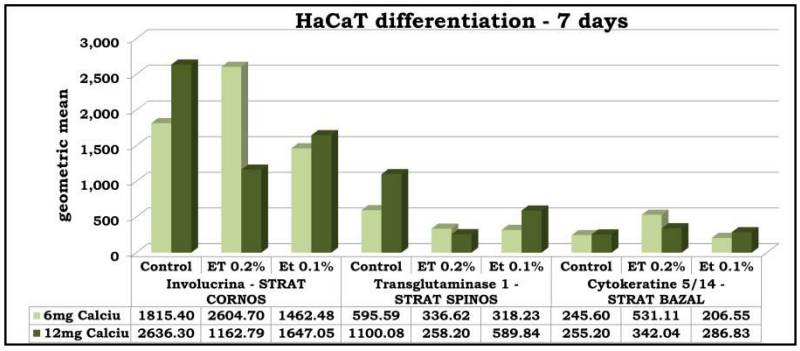


	Control	TES 0,5mg/ml	TES 1mg/ml	TES 2mg/ml	Control	TES 0,5mg/ml	TES 1mg/ml	TES 2mg/ml	Control	TES 0,5mg/ml	TES 1mg/ml	TES 2mg/ml
	Concentratie mica de calciu				Concentratie medie de calciu (6mg)				Concentratie mare de calciu (12mg)			
Faza S%	6.25	7.12	6.3	6.78	10.91	10.51	12.78	12.72	3.43	5.71	6.98	6.69
G2/M%	10.08	9.19	11.13	10.81	8.86	8.56	7.64	7.71	5.11	5.18	5.67	6.23

Figure 35. Effect of **TES** extract on cell cycle sequence at different calcium concentrations

TES grape extract stimulates the expression of cytokeratin, inducing keratinocyte differentiation from the basal layer, without selectivity in terms of calcium intake in the extracellular environment. This suggests a role in inducing intrinsic regeneration, independent of external differentiation promoters. There is also the overexpression of transglutaminase-1, so the progression of the spinous layer, but also of the involucrin, characteristic of the completely differentiated corneum layer, both especially in the conditions of an extracellular calcium intake. **TES** stimulates the turnover of keratinocytes both in undifferentiated conditions (low in calcium) and induction of differentiation (12 mg of calcium), an effect that supports the complete process of epidermal regeneration.

In order to highlight in vitro this process of epidermal renewal, several differentiation markers belonging to different evolutionary stages were analyzed in the presence of **ET** extract: keratin 5/14 - molecule that is expressed only in the basal layer; transglutaminase-1 in the spinous layer and involucrin in the granular layer, the stratum corneum being formed only by anucleated, non-viable cells.



	Control	ET 0.2%	Et 0.1%	Control	ET 0.2%	Et 0.1%	Control	ET 0.2%	Et 0.1%
	Involucrina - STRAT CORNOS			Transglutaminase 1 - STRAT SPINOS			Cytokeratine 5/14 - STRAT BAZAL		
6mg Calciu	1815.40	2604.70	1462.48	595.59	336.62	318.23	245.60	531.11	206.55
12mg Calciu	2636.30	1162.79	1647.05	1100.08	258.20	589.84	255.20	342.04	286.83

Figure 36. Highlighting the differentiation process in keratinocytes treated with **ET** red clover extract

ET extract stimulates their differentiation due to the positive impact on the expression of all molecular markers analyzed in different stages of skin cornification. Thus, an important contribution of phytocompounds to the epidermal renewal process and implicitly to the prevention and slowing of the progression of degenerative skin aging processes, as well as post-traumatic recovery in lesions of various etiologies (burns, wounds, skin infections, etc.).

B. Studies on the homeostasis of the extracellular matrix secreted by dermal fibroblasts

Controlled degradation of the ECM is a necessary process for cell migration, angiogenesis, reepithelialization, temporary matrix degradation, and remodeling of newly formed granular tissue during wound healing. ECM is composed of a complex mixture of insoluble molecules including collagen, laminins, fibronectin, entactin / nidogen and heparin-sulfate proteoglycans, providing a solid support for cells. ECM also includes

embedded cytokines and growth factors, as well as communication pathways between the molecules that make up the ECM network, facilitating cell migration, adhesion, wound contraction and epithelialization. Turnover and ECM remodeling must be strictly regulated because uncontrolled proteolysis contributes to abnormal development and generates many pathological conditions characterized by excessive degradation, such as chronic ulcers or lack of degradation of ECM components leading to fibrosis [Meilang et al., 2006, Eckes et al., 2010].

Healing of skin lesions is a complex physiological process, which involves coordinated interactions between different biological and immunological systems, involving a cascade of steps and very precisely organized events, which correlate with the appearance of certain cell types at the site of affected tissue during different phases. of the healing process [Velnar et al., 2009]. Skin lesion repair can be divided into several dynamic stages including: (i) fibrin clot formation and inflammatory response; (ii) granular tissue formation that includes reepithelialization and angiogenesis; and (iii) formation and remodeling of the extracellular matrix. A dynamic balance between endothelial cells, platelets, coagulation and fibrinolysis regulates hemostasis and determines the amount of fibrin stored at the wound site, thus influencing the progress of repair processes [Meilang et al., 2006, Velnar et al., 2009].

To restore the dermal layer, it is necessary for fibroblasts to synthesize collagen fibers, elastic fibers and the components of the base substance, but also to establish an optimal activation / inhibition ratio for the enzymes involved in matrix remodeling. Under the action of growth factors, (especially TGF-β), fibroblasts secrete collagen in the extracellular space for the formation of granular tissue. Collagen fibers, found in granular tissue, are type I and III, and in the healing tissue resulting from the matrix remodeling process, type I collagen fibers are mainly found [Nguyen et al., 2009].

The process of repairing damaged tissues is controlled by certain regulatory mechanisms in order to maintain the balance between metabolism and catabolism, ultimately leading to normal healing. Matrix metalloproteinases (MMPs), produced by neutrophils, macrophages and fibroblasts, are directly involved in the process of collagen degradation, their activity being closely linked and regulated by inhibitory agents [Velnar et al., 2009]. MMPs are not constitutively expressed in the skin but are temporarily induced in response to exogenous signals such as cytokines, growth factors, cell-matrix interactions, or cell-cell interactions. In the process of dermal recovery, MMPs are involved in the removal of devitalized tissue, epidermo-mesenchymal interactions during keratinocyte migration, angiogenesis, remodeling of newly synthesized connective tissue during maturation, regulation of the activity of certain growth factors [Kahari et al.].

Regulation of the extracellular matrix implies a balance between the synthesis of its structural components and their degradation under the catalytic action of MMPs whose biological function is modulated by specific tissue inhibitors of matrix metalloproteinases (TIMPs). Overregulation of MMP activity favors proteolytic degradation of the basement membrane and extracellular matrix, being correlated with tumor growth and metastasis, as well as tumor-associated angiogenesis, while inhibition of MMP activity appears to restrict these processes [Kahari et al., 1997].

MMPs are responsible for the extracellular matrix proteins degradation (ECMs) such as collagen, fibronectin, elastin and proteoglycans, contributing to the photoaging process. [Cavinato et al., 2017]. MMP-9 (gelatinase B), is produced by human keratinocytes and acts on type IV collagen, an important component of the basement membrane in the skin. Like MMP-9, MMP-2 (gelatinase A) is able to cleave type IV collagen. In addition, both gelatinases can degrade other substrates such as type V, VII and X collagen, fibronectin and elastin. They are essential in the degradation of fibrillar collagen fragments after their initial degradation by collagenases [Pittayapruek et al., 2016]. During the aging process, extracellular matrix proteins are susceptible to excessive activity of proteolytic enzymes-matrix metalloproteinases (MMPs), being mostly bound to collagen and elastin. Under appropriate physiological conditions, the enzymes are regulated at the transcriptional level and by protein inhibitors. Imbalance in homeostasis leads to loss of tissue integrity, which can lead to loss of skin elasticity.

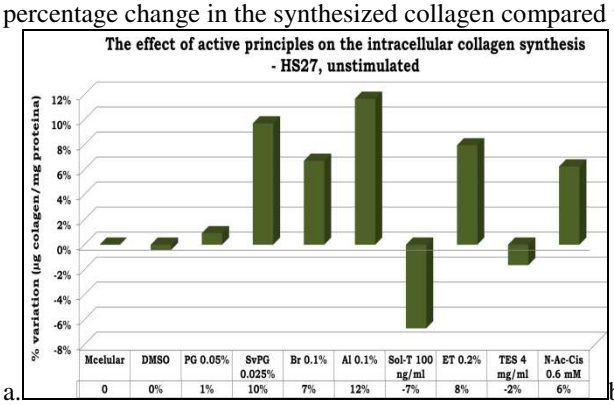
Over time, numerous studies have been conducted on the role of plant extracts as skin tissue repair agents. These bioactive compounds such as alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins and phenolic compounds are known to be involved in various phases of the healing process, and due to their multifunctional properties (anti-inflammatory, antioxidant, etc.) [Tsala et al., 2013].

To evaluate the role of plant extracts in the dermal remodeling process, the relevant methods described in the dedicated chapter were used. The evaluation of the collagen synthesis process in the presence of principles was performed by determining the total intracellular collagen (by labeling with the Red / Fast Green Sirius kit - method 4.2.8.). The evaluation of matrix metalloproteinases secreted in the growth environment by dermal fibroblasts was performed by zymography (method 4.2.9.) [Craciun et al., 2019].

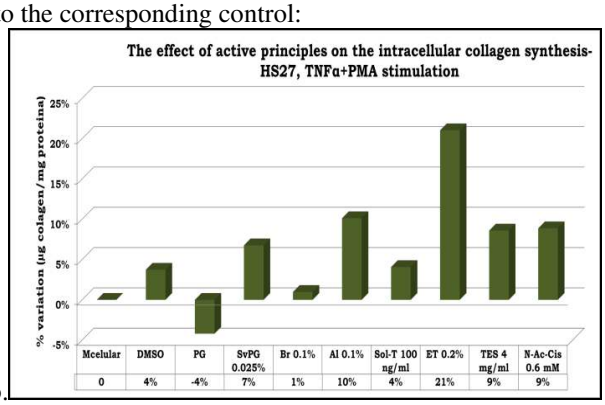
Determination of total collagen - labeling with Sirius Red / Fast Green

Fibroblasts (HS27) were cultured for 24 h in 24-well plates in DMEM culture medium, supplemented with 10% fetal bovine serum and 1% antibiotic. The cells were treated for 48 hours, of which 24 hours with TNFα and PMA stimuli, respectively LPS and PMA. After 48 hours, the culture medium is collected (the activity of matrix metalloproteinases secreted in the extracellular environment will be determined). The results are plotted as a percentage change in the synthesized collagen compared to the corresponding control:

a.



b.



c.

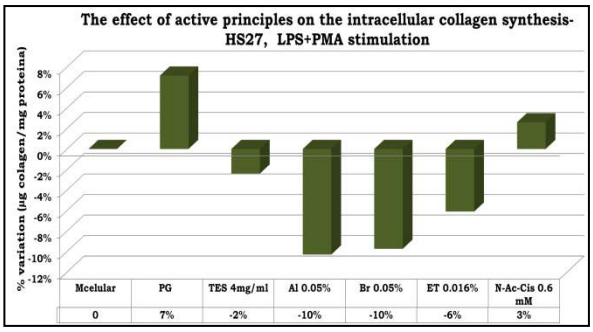


Figure 37. Intracellular collagen dosing with the Sirius Red / Fast Green kit - dermal fibroblasts treated with **SvPG, Br, Al, Sol-T, ET** and **TES**, under normal conditions (a.) And with proinflammatory stimulation (TNFα) associated with endogenous oxidative stress (PMA) - (b.) and bacterial associated with oxidative stress (LPS + PMA) - (c.)

Identification and assay of matrix metalloproteinases

It is a method of estimating the concentration of gelatinases (MMP-2 and MMP-9) in the conditioned environment based on the ability of these enzymes to regenerate after electrophoretic migration in gelatin copolymerized polyacrylamide-SDS gels and removal of SDS by repeated washings. with Triton X-100, the enzymes thus exerting their proteolytic activity on the copolymerized substrate during 18 hours of incubation at 37 ° C in a suitable buffer. The zymograms were scanned and analyzed semi-quantitatively with ImageJ software by densitometry of protein bands with enzymatic activity that appear as lysis beaches, and the identification of the type of MMP was made based on molecular masses.

The expression of matrix metalloproteinases in the presence of **SvPG, Br, Al, Sol-T, ET** and **TES** extracts is shown in the figure below:

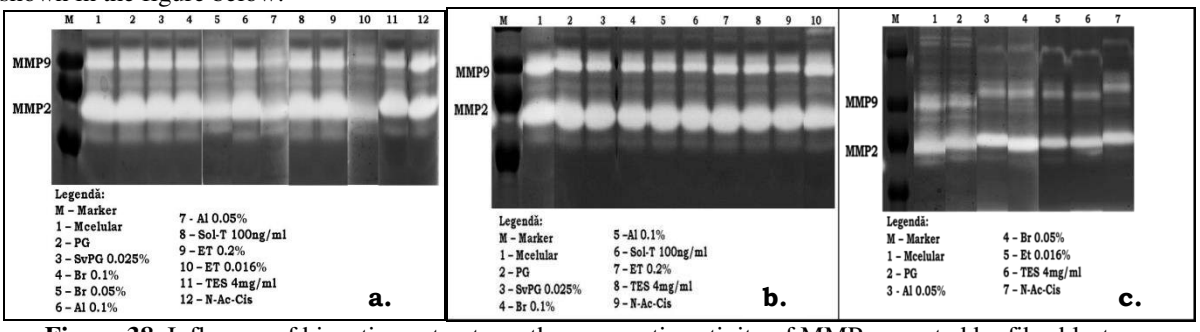


Figure 38. Influence of bioactive extracts on the enzymatic activity of MMPs secreted by fibroblasts: a. Unstimulated; b. stimulated with TNFα + PMA and c. stimulated with LPS + PMA

In this experimental model, the effect of bioactive extracts of *Salvia officinalis (**SvPG**), Solanum lycopersicum (**Sol-T**), Corylus avellana (**Al**), Arctium lappa (**Br**), Trifolium pratense (**ET**)* and *Vitis vinifera (**TES**)*

was evaluated on the skin regeneration process by monitoring two important phenomena at the dermo-epidermal level, respectively keratinocyte differentiation and matrix remodeling at the level of dermal fibroblasts.

Relevant physiological mechanism: Skin regeneration by / Plant source / extract		**Keratinocyte differentiation**	**Collagen synthesis**	**MMP**
Salvia officinalis	**SvPG**		stimulates collagen synthesis - 7%	↓↓↓
Arctium lappa	**Br**		stimulates collagen synthesis - 12%	↓↓↓↓
Corylus avellana	**Al**		stimulates collagen synthesis - 7%	↓↓↓↓
Solanum lycopersicum	**Sol-T**	overexpression of transglutaminase-1.7 days; epidermal regenerative effect	does not significantly influence the process of collagen synthesis	↓↓↓
Trifolium pratense	**ET**	regenerative effect	stimulates collagen synthesis - 21%	↓↓↓↓
Vitis vinifera	**TES**	stimulates keratinocyte turnover; complete regenerative effect	stimulates collagen synthesis - 9%	↓↓↓↓

From the analysis of the experimental data, both in conditions of induction of differentiation (by calcium intake in the extracellular environment) and in calcium-poor environment, there is an important intake of phytocompounds tested in the renewal process of the epidermis and implicitly in prevention and slowing progression of degenerative processes of skin aging, as well as post-traumatic recovery in lesions of various etiologies (burns, wounds, skin infections, etc.). Among the extracts tested, it can be noticed: the **Sol-T** extract mainly induces the overexpression of transglutaminase-1, so the progression of the spinous layer; **TES** grape seed extract stimulates the expression of cytokeratin, inducing keratinocyte differentiation from the basal layer and overexpression of transglutaminase-1, so the progression of the spinous layer, but also of the involucre, characteristic of the completely differentiated corneum layer; **ET** extract stimulates keratinocyte differentiation due to the positive impact on the expression of all molecular markers analyzed in different stages of skin cornification.

Among the bioactive phytocompounds tested, **ET** and **TES** extracts show a significant stimulating effect of collagen synthesis in dermal fibroblasts, both in basal conditions and in nonspecific stimulation conditions with TNFα and PMA, thus contributing to the dermal regeneration process.

Matrix metalloproteinases are of particular importance in tissue remodeling and wound healing, but are also an important element in the progression of inflammatory diseases and tumor invasion (proteolytic activity of protein enzymes being correlated with the metastatic potential of tumor cells). Following the evaluation of the enzymatic activity of matrix metalloproteinases secreted in the extracellular culture medium by dermal fibroblasts, in the presence of bioactive extracts, the following are noted: **ET** and **TES** extracts act on metalloproteinases to decrease their activity both in basal conditions and after stimulation with proinflammatory and bacterial agents. The same inhibitory effect on metalloproteinases is shown by the Sol-T extract, which intervenes more pronounced on MMP9, reducing its activity by up to 27%. The same inhibitory effect is observed in the case of proinflammatory and bacterial stimulated cells, and treated with burdock (**Br**) and hazelnut (**Al**) extracts.

5.7. Mechanisms involved in melanomic progression modulated by combinations of biologically active phytocompounds from red clover and grape waste

The incidence of skin cancer is based on a number of causative agents, the most important of which is UV radiation, but also certain viruses, mutagenic compounds in food or chemicals, genetic susceptibility, play an equally important role in the occurrence of this diseases. The appearance of skin cancer can be prevented by eliminating these external factors and can be effectively removed by preventing the blood supply to the tumor (anti-angiogenesis), stopping tumor growth and thus increasing the patient's survival rate. Most cancer cells develop ways to prevent apoptosis or have defective apoptosis mechanisms, thus allowing uncontrolled cell development. Malignant melanoma of the skin is the most serious form of skin cancer, occurs in epidermal melanocytes and is a malignancy refractory to treatment and metastasis, the incidence of which has steadily and significantly increased in recent decades [D'Orazio et al., 2013].

In human melanoma cell culture, increased expressions of MMP-1, MMP-2 and MMP-9 have been shown to be correlated with migration and tumor invasion. In human melanocyte lesions, a positive correlation was demonstrated between tumor progression and MMP-2 expression. Increased MMP-9 expression, on the other hand, was found mainly in the radial growth phase of primary melanoma, indicating that MMP-9 expression correlates with early melanoma invasion [Neufeld et al., 1994].

The severe side effects of chemotherapeutic agents as well as the development of a resistance of the body to several drugs used in cancer therapy, have led to the development of strategies to eliminate them, strategies that address the use of nanoparticles, liposomes and mycelial release vehicles. reported [Iyer et al., 2013]. Due to these undesirable effects associated with conventional cancer therapy, over time the need for alternative treatment schemes in which active principles from natural sources have been preferred over synthetic ones has become increasingly important.

Phytochemicals that have anti-inflammatory, immuno-modulatory and anti-oxidant properties generally have the greatest potential to exhibit chemo-preventive behaviors in skin cancers. Numerous attempts have been made to find a correlation between the antioxidant properties of phytocompounds and their anti-cancer potential. Although no concrete evidence of such a correlation has been found, the antioxidant activity of a phytocompounds is considered an indication of potential anticancer activity. Carotenoids, flavonoids and terpenoids are some of the groups of phytocompounds with high anticancer potential [Agrawal, 2011].

Resveratrol (a compound found in significant amounts in grapes) has been investigated as an anti-cancer agent and has been found to be able to inhibit the growth of melanomic and amelanomic cells by inducing apoptosis. The potency of resveratrol has been demonstrated by its ability to induce apoptosis in doxorubicin-resistant murine melanoma cells and its ability to inhibit the growth of doxorubicin-resistant melanoma tumors in mice. Resveratrol has anti-metastatic potential because it has been reported to inhibit the lipopolysaccharide-induced epithelial transition to the mesenchymal transition, possibly by inhibiting NF-κB signaling. There is also the potential to apply resveratrol as a radiation sensitizer in the treatment of melanoma, as it has been observed that radiation-resistant melanoma cells have responded well to a combination of resveratrol and radiation treatment [Niles et al., 2003].

Red clover (*Trifolium pratense*) is a medicinal plant traditionally used in the treatment of chronic skin conditions, containing at least four estrogenic isoflavones: formononetin, biochanine A, daidzein and genistein. In addition to their estrogenic activity, phytoestrogens also have nonhormonal activities such as antioxidant but are also potential antitumor agents. For example, the anticarcinogenic effect of genistein has been demonstrated in vitro, probably due to its inhibitory activity on protein tyrosinase kinase and angiogenesis [Kolodziejczyk-Czepas, 2012].

The target mechanisms studied in order to complete the cytotoxicity / efficacy profile are: melanin synthesis as an indicator parameter of malignancy; tumor invasiveness expressed by metalloproteinase activity (MMP 2 and MMP 9); intercellular signaling by pro-angiogenic factor VEGF and cytokine IL6; proliferative and pro-apoptotic status in melanoma under the conditions of UV irradiation (methods described in the dedicated special chapter) [Christmas et al., 2018].

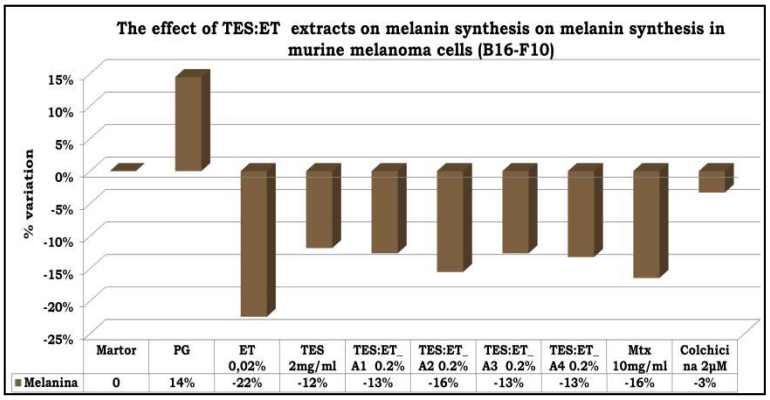
The standardized cell line used is that of murine melanoma - B16-F10 - with relevance and predictability in human pathology. Cells were cultured in DMEM culture medium with 10% fetal bovine serum, 1% antibiotic / antifungal solution, under standard conditions (37 ° C, 95% humidified air and 5% CO2) 24 hours before treatment and 48 hours with tested substances.

The two test extracts were combined in the following proportions: TES: ET_A1 = 1: 9; TES: ET_A2 = 1: 5; TES: ET_A3 = 1: 3; TES: ET_A4 = 2: 9. The experiments were performed in triplicate, the data presented being the average of the values obtained in the 3 successive experimental series, having as positive controls: Methotrexate 10mg / ml - broad-spectrum antiproliferative agent; Colchicine 2μM - active antitumor in blocking the cell division cycle.

Evaluation of melanin as an indicator of malignancy:

Melanin pigments are produced in mammals especially as protection against UV radiation, especially UV-B, directly absorbed by cellular DNA. UV-A radiation acts mainly by photosensitization, generating radical species that degrade DNA and other cellular components. UV-A penetrates the dermis, deeper than UV-B, being the major source of UV radiation responsible for the production of certain types of skin cancer [Chiarelli-Neto et al., 2014; Jimbow et al., 1993].

The relevant results for the action of **TES** and **ET** compounds, singular and associated in the selected proportions are presented in the table below:

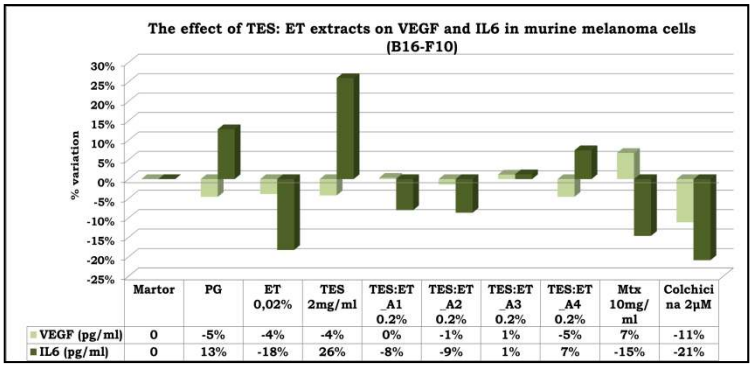


	Martor	PG	ET 0,02%	TES 2mg/ml	TES:ET_ A1 0.2%	TES:ET_ A2 0.2%	TES:ET_ A3 0.2%	TES:ET_ A4 0.2%	Mtx 10mg/ml	Colchicina 2µM
Melanina	0	14%	-22%	-12%	-13%	-16%	-13%	-13%	-16%	-3%

Figure 39. Determination of melanin secretion secreted by B16-F10 cells in the presence of **TES: ET** combinations. The absorbance of melanin pigments is read at 450nm.

Evaluation of invasiveness indicators: VEGF, IL6, Metalloproteinases (MMP)

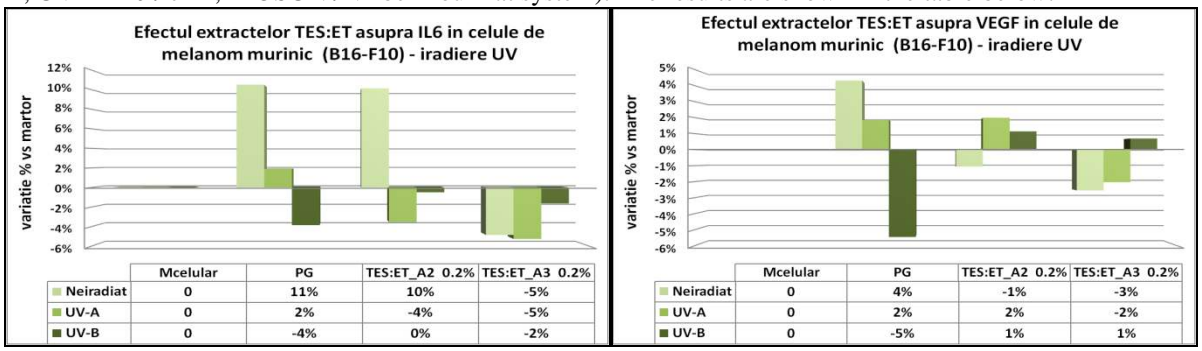
Screening for pro-angiogenic factor VEGF and cytokine IL6 was performed initially under basal conditions of culture development of melanoma in the B16-F10 cell line, as described above. The results are presented in the table below:



	Martor	PG	ET 0,02%	TES 2mg/ml	TES:ET _A1 0.2%	TES:ET _A2 0.2%	TES:ET _A3 0.2%	TES:ET _A4 0.2%	Mtx 10mg/ml	Colchicina 2µM
VEGF (pg/ml)	0	-5%	-4%	-4%	0%	-1%	1%	-5%	7%	-11%
IL6 (pg/ml)	0	13%	-18%	26%	-8%	-9%	1%	7%	-15%	-21%

Figure 40. Determination of VEGF and IL6 in the B16-F10 cell line in the presence of **TES: ET** combinations.

Exposure of the cell culture to UV-A and UV-B radiation was performed in controlled doses (UV-A - 10J / cm2; UV-B - 1J / cm2, BIOSUN / Vilber Lourmat system). The results are shown in the table below:



	Mcelular	PG	TES:ET_A2 0.2%	TES:ET_A3 0.2%
Neiradiat	0	11%	10%	-5%
UV-A	0	2%	-4%	-5%
UV-B	0	-4%	0%	-2%

	Mcelular	PG	TES:ET_A2 0.2%	TES:ET_A3 0.2%
Neiradiat	0	4%	-1%	-3%
UV-A	0	2%	2%	-2%
UV-B	0	-5%	1%	1%

Figure 41. Determination of VEGF and IL6 in the non-irradiated B16-F10 / UV-A / UV-B cell line in the presence of **TES: ET_A2** and **TES: ET_A3** extracts.

The TES: ET_A3 mixture intervenes only under the conditions of UV-A irradiation in the tumor propagation cascades coordinated by IL6, by inhibiting them, the extracts not being active after UV-B irradiation.

Evaluation of the effect of **TES: ET** extracts on the enzymatic activity of MMPs secreted in culture medium B16-F10:

Skin melanoma is the most common malignant tumor in young people and is characterized by a high capacity for invasion and metastasis. In this process the degradation of the ECM and the basement membrane with proteolytic enzymes is an essential step, in which MMP plays an important role. Overexpression, MMP-2 and MMP-9 has been implicated in the migration and invasion of melanoma cells. Increased activity of MMP-2 has been shown to be associated with tumor progression in several types of tumors (brain tumors, gastric carcinoma). In melanocyte lesions, a correlation was found between the expression of MMP-2 metalloproteinase and the weakening

of the architectural structure, atypical growth and hematogenous metastases. Unlike MMP-2, which was associated with melanoma progression, MMP-9 was expressed only in advanced primary melanomas and was absent in the cell lines from the early stage of primary lesions. [Hofmann et al., 2000].

Following electrophoretic migration, the zymograms were scanned and analyzed semi-quantitatively with ImageLab software by densitometry of protein bands with enzymatic activity that appear as lysis beaches, and the identification of the type of MMP was made based on molecular masses. The results are shown in the graphs below, as a percentage change from the corresponding control.

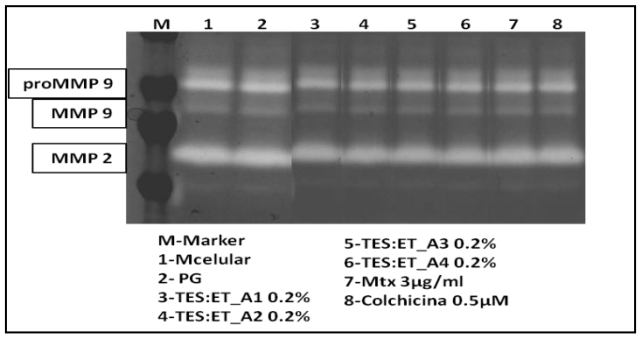


Figure 42. Determination of MMP activity in the non-irradiated B16-F10 cell line in the presence of **TES: ET** combinations.

Under the conditions of UV irradiation, the enzymatic activity of MMP secreted in the culture medium by melanoma cells, two of the four combinations of extracts were tested, namely **TES: ET_A2 and TES: ET_A3**. Zymograms and quantification of lysis bands are shown in the figure below:

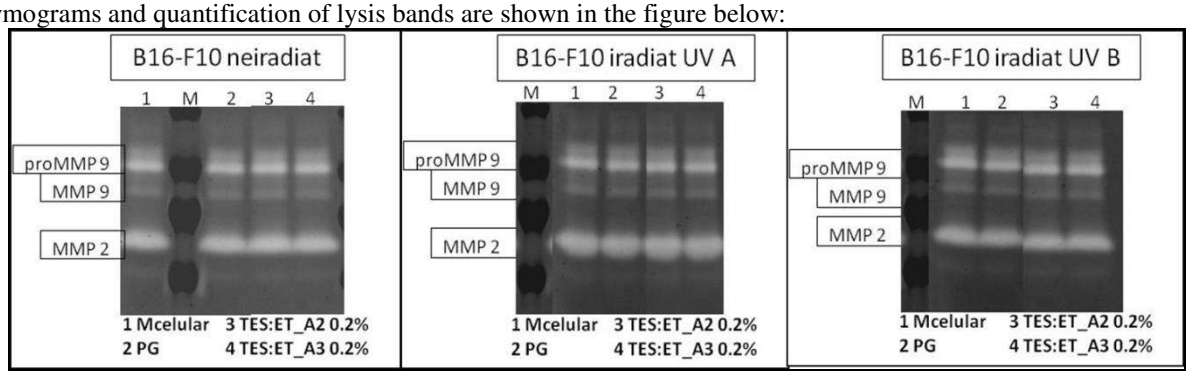
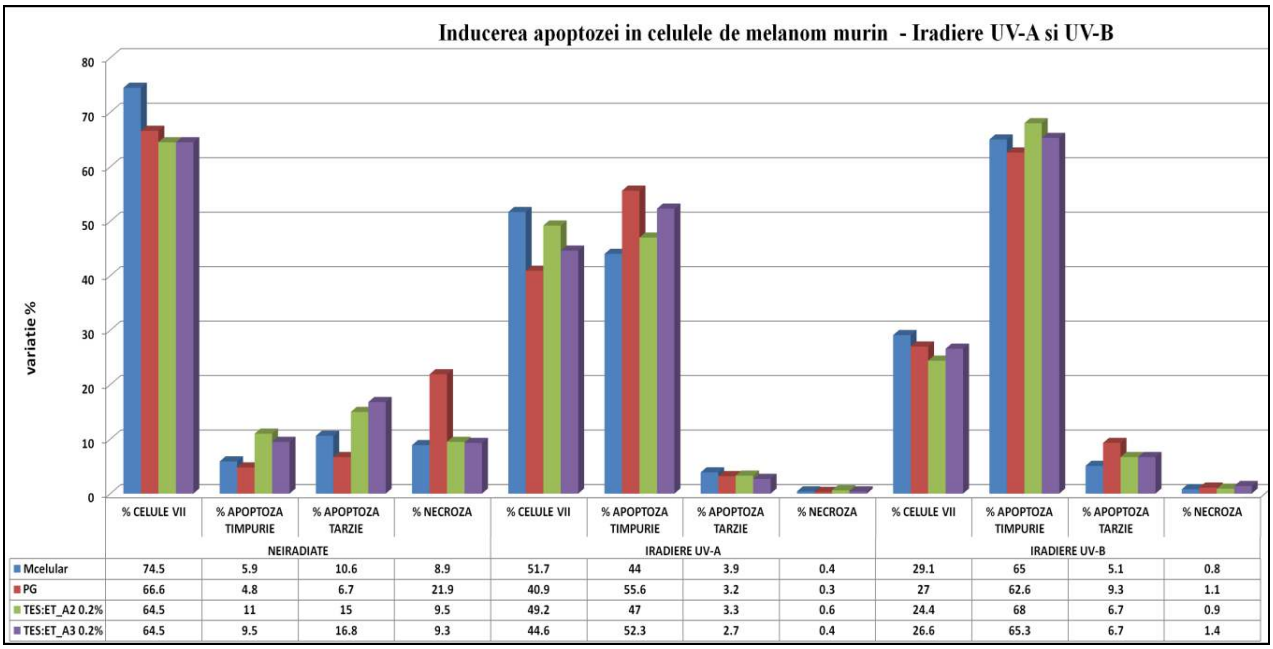


Figure 43. Determination of MMP activity in B16-F10 cell line irradiated with UV-A / UV-B in the presence of TES: ET combinations

Proliferative and pro-apoptotic status in melanoma under UV irradiation conditions

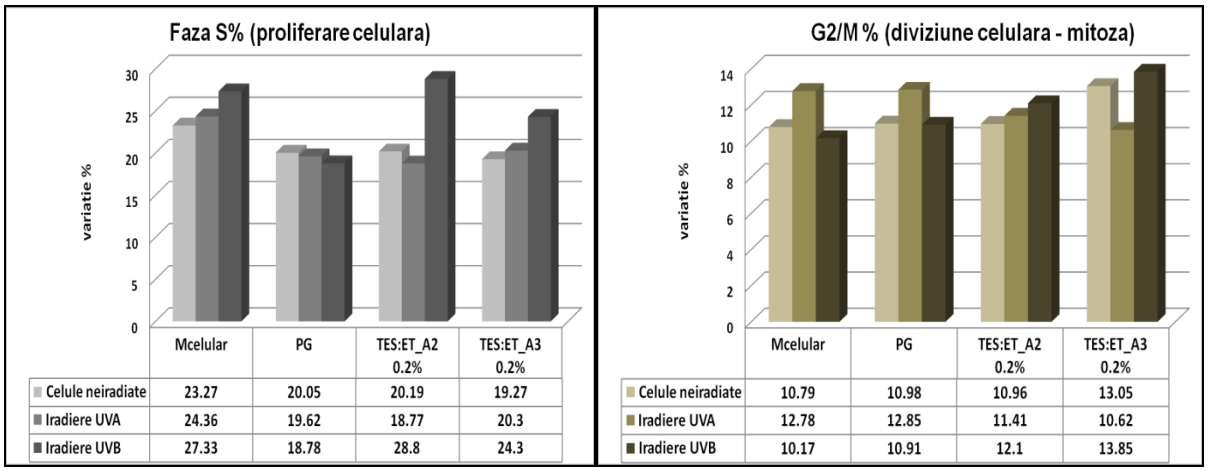
The studies aimed to highlight the main processes involved in tumor progression (apoptosis and cell cycle sequencing) in the murine melanoma cell line B16-F10, in an experimental model of UV irradiation. The mechanisms followed in testing these extracts, within the irradiation model, were the following: evaluation of the apoptotic process; evaluation of the proliferative status by highlighting the successive generations and the sequentiality of the cell cycle.

We performed 3 experimental series consisting of unirradiated B16-F10 cells, irradiated UV-A (9J / cm2) and UV-B (1J / cm2). The cells were allowed to adhere for 24 hours, then treated with the extracts for 6 hours, irradiated and cultured for another 24 hours in the presence of the extracts. Apoptosis and cell cycle sequentiality were analyzed according to the methods described in the dedicated chapter. The results are presented in the tables below:



	% CELULE VII	% APOPTOZA TIMPURIE	% APOPTOZA TARZIE	% NECROZA	% CELULE VII	% APOPTOZA TIMPURIE	% APOPTOZA TARZIE	% NECROZA	% CELULE VII	% APOPTOZA TIMPURIE	% APOPTOZA TARZIE	% NECROZA
	NEIRADIATE				IRADIERE UV-A				IRADIERE UV-B			
Mcelular	74.5	5.9	10.6	8.9	51.7	44	3.9	0.4	29.1	65	5.1	0.8
PG	66.6	4.8	6.7	21.9	40.9	55.6	3.2	0.3	27	62.6	9.3	1.1
TES:ET_A2 0.2%	64.5	11	15	9.5	49.2	47	3.3	0.6	24.4	68	6.7	0.9
TES:ET_A3 0.2%	64.5	9.5	16.8	9.3	44.6	52.3	2.7	0.4	26.6	65.3	6.7	1.4

Figure 44. Apoptosis of B16-F10 murine melanoma cells in the presence of **TES: ET**



	Mcelular	PG	TES:ET_A2 0.2%	TES:ET_A3 0.2%
Celule neiradiate	23.27	20.05	20.19	19.27
Iradiere UVA	24.36	19.62	18.77	20.3
Iradiere UVB	27.33	18.78	28.8	24.3

	Mcelular	PG	TES:ET_A2 0.2%	TES:ET_A3 0.2%
Celule neiradiate	10.79	10.98	10.96	13.05
Iradiere UVA	12.78	12.85	11.41	10.62
Iradiere UVB	10.17	10.91	12.1	13.85

Figure 45. Sequence of the cell cycle under UV-A and UV-B irradiation conditions on the murine melanoma line B10-F16

The sequence of the cell cycle does not change under the tested irradiation conditions, maintaining the same accelerated multiplication rate specific to melanoma. The A2 and A3 type combinations provide a concerted effect of reducing tumor spread on the two mechanisms mentioned above. Under irradiation conditions, the extracts act only in the case of UV-A, maintaining the trend observed in the basal stage. UV-B radiation is much more aggressive, inducing apoptosis in most cell populations, by generating reactive oxygen species.

In human melanoma cell culture, increased expressions of MMP-1, MMP-2 and MMP-9 are correlated with migration and invasion. In human melanocyte lesions, a positive correlation was demonstrated between tumor progression and MMP-2 expression. Increased MMP-9 expression, on the other hand, was found mainly in the radial growth phase of primary melanoma, indicating that MMP-9 expression correlates with early melanoma invasion [Neufeld et al., 1994]. Under conditions of UV irradiation, a more pronounced inhibitory effect is observed on MMP 9 and MMP 2, in the case of cells treated with the two mixtures of extracts, attenuating the invasive character of murine melanoma.

The studies also aimed to highlight the main processes involved in tumor progression (apoptosis and cell cycle sequencing) in the murine melanoma cell line B16-F10, in an experimental model of UV irradiation. The **TES: ET_A2** and **TES: ET_A3** mixtures provide a concerted effect of reducing tumor spread on the two

mechanisms mentioned above. Under irradiation conditions, the extracts act only in the case of UV-A, maintaining the trend observed in the basal stage. UV-B radiation is much more aggressive, inducing apoptosis in most cell populations, by generating reactive oxygen species.

FINAL CONCLUSIONS

The research undertaken for the doctoral thesis approached a complex study on the action of plant extracts rich in polyphenols, triterpene acids, polyphenylcarboxylic acids, polyacetylenes, anthocyanins, flavones, steroidal glycaloalkaloids, and phytoestrogens, on skin relevant cellular systems, in respect with homeostatic restoring of physiological and pathological processes disturbed by free radicals actions. For an objective result of the research data, the algorithm of tests and analyzes performed in this thesis took into account a screening of parameters relevant to the pro and anti-oxidant system. The results obtained add value to the products know-how and associated mechanisms for increased therapeutic potential in order to capitalize on the natural compounds *of Salvia officinalis, Arctium lappa, Corylus avellana, Trifolium pratense* and vegetable waste from *Solanum lycopersicum* and *Vitis vinifera* species.

Therefore, significant *in vitro* experimental models have been developed to describe the physiological and pathological processes within the dysfunctional cellular status regarding the generation of free radicals in situ for acellular evaluations; differentiated, pathological-specific pro-inflammatory stimulation: TNF-α (systemic localization, first line of generation of inflammation propagation cascades), PMA (pro-oxidant, inflammation promoter), LPS (bacterial infection in skin lesions); expression of monocyte-endothelium adhesion molecules and correlations with cytokine release in vascular inflammation; pro-oxidative and pro-inflammatory phenomena generated by UV radiation with an impact on proliferation and apoptosis at the level of normal cell - fibroblast, respectively melanoma; post-lesional dermo-epidermal regeneration, expressed by keratinocyte differentiation and optimal ratio of collagen synthesis / degradation.

The efficacy / toxicity profile of the biocomplexes studied and subjected to multifactorial investigation in specific cellular and molecular events / processes resulted from advanced analyzes of the interdependent relationships of *in vitro* effects, synthesizing the following:

A. General screening, at acellular and cellular level, on the antioxidant / antiradical action of plant extracts:

- The 3 correlative methods for evaluating the antioxidant / antiradical capacity, applied in the primary antioxidant screening highlighted their performance in the developing of in vitro experimental models and the ability of the studied biocomplexes (**SvPG, Al, Br, ET, TES**) to capture free radicals, directing their further use. They act to modulate the main metabolic pathways to counteract reactive oxygen species involved in the initiation and spread of various pathologies (atherosclerosis, cancer, diabetes, liver disease, inflammation, skin lesions, arthritis, etc.).
- **At the intracellular level, in Superoxide dismutase / Catalase enzyme system** that generate and transform reactive oxygen species, the biocomplexes **Sol-T, TES, Al** and **Br**, act on the activity of enzymes involved in endogenous antioxidant cascade counteracting free radical attack, eliminating superoxide anions and peroxides**.**
- **SvPG** and **Sol-T** extracts demonstrate a **pronounced antioxidant effect at the dermo-epidermal level**, by reducing the oxidative species ($\bullet O_2^-$.and H_2O_2), **contributing to cellular protection in skin inflammation associated with bacterial infections**. In case of mimicking conditions of endogenous oxidative stress (PMA) simultaneously with bacterial inflammation (LPS), the level of intracellular ROS is strongly low in the presence of **Al extract, showing a protective effect against bacterial attack associated with skin conditions (burns, open wounds, acne, etc.)**.
- **SvPG sage extract inhibits the production of reactive oxygen species in endothelial cells** stimulated with TNFα and PMA, the effect being comparable to that of the positive control tested under the same experimental conditions. **Sol-T** glycaloalkaloid extract indicates a reducing effect on the superoxide anion under both stimulation conditions, but generates an accumulation of hydrogen peroxide in the system, which may result in a change in vascular reactivity and lead to toxicity and alterations in homeostasis at the vascular level.

B. Impact of plant complexes on the **inflammatory status of the skin tissue** by simulating inflammatory aggressions (fungal or bacterial attack, respectively nonspecific inflammation):

- **Sol-T** glycoalkaloid extract inhibits the extracellular release of cytokines IL6, IL8, IL1α, demonstrating anti-inflammatory and anti-irritant effect by blocking the signaling pathways coordinated by these cytokines. Decreasing the pro-angiogenic factor VEGF released by keratinocytes under TNFα stimulation and increasing it under the action of bacterial LPS recommends this compound in dermatological

conditions without active lesions (eg dermatitis, skin irritations, etc.), respectively healing of superinfected wounds (induces microvascular formation).

- **SvPG** sage extract stops the extracellular signaling generated by the cytokine IL6, relevant even in serious dermatological diseases such as psoriasis.
- At the dermal fibroblasts level, under the action of a bacterial stimulus associated with an oxidative stress generating stimulus, **Al** extract acts on proinflammatory cytokines, in the sense of their inhibition, demonstrating a pronounced anti-inflammatory effect.
- **Sol-T** extract has a significant effect in conditions of nonspecific inflammation induced by TNFα, acts by reducing the extracellular expression of cytokines IL6 and IL8, thus manifesting an anti-inflammatory effect in the endothelial cell. The bacterial attack at the endothelial level (stimulation with LPS) is counteracted by the glycoalkaloid extract **Sol-T** which has a reducing effect on the expression of ICAM, a characteristic parameter of small blood vessels.

C. Photoprotective effect of the active principles of *Salvia officinalis* (SvPG) and *Corylus avellana* (Al) studied by modulating dermo-epidermal cellular mechanisms under the influence of UV radiation:

- The active principles extracted from sage (**SvPG**), act by preventing the progression of early apoptosis in cells irradiated with UV-A or UV-B. **SvPG** sage extract is particularly active in reducing oxygen free radicals.
- **Al** extract, has the effect of intracellular reduction of reactive oxygen species especially in conditions of UV-B irradiation, induces a decrease in inflammatory phenomenon and also has an antiangiogenic action in the case of UV-A radiation.

D. Effect of bioactive extracts of *Salvia officinalis* (**SvPG**), *Solanum lycopersicum* (**Sol-T**), *Corylus avellana* (**Al**), *Arctium lappa* (**Br**), *Trifolium pratense* (**ET**) and *Vitis vinifera* (**TES**) on the **skin regeneration process**:

- In conditions of induction of differentiation (by calcium intake from the extracellular environment), as well as in calcium-poor environment, there is an important involvement of phytocompounds from **Sol-T, ET** and **TES** extracts in the epidermal renewal process, to prevent and slow down the progression of degenerative processes of skin aging, as well as post-traumatic recovery in injuries of various etiologies (burns, wounds, skin infections, etc.).
- Among the bioactive phytocompounds tested, **ET, TES, Al** and **Br** extracts show a significant **stimulating effect of collagen synthesis in dermal fibroblasts**, both in basal conditions and in conditions of nonspecific stimulation with TNFα and PMA, contributing to the process of dermal regeneration.
- The activity of MMP in the culture of dermal fibroblasts decreases in the presence of bioactive extracts **ET, TES, Al** and **Br**, demonstrating a special importance of these biocomplexes in tissue remodeling and wound healing, but also in the progression of inflammatory diseases and tumor invasion.

E. The experimental model developed for the study of **cellular and molecular processes in the pathology of hyperproliferative disorders (melanoma) was performed by testing the associated TES and ET biocomplexes, both under normal conditions and after UV irradiation, on the standardized cell line of murine melanoma B16 -F10, noting the following:**

- The association of the biocomplex with strong antioxidant effect **TES**, with the phytoestrogenic extract **ET**, reduces the amount of melanin (main indicator of malignancy in skin cancers) produced by melanoma cells.
- Screening at the level of pro-angiogenic factor VEGF and cytokine IL6 demonstrated the inhibitory effect of the mixture TES: ET_A2, on melanoma cells in basal conditions of development. Under the conditions of UV-A irradiation, the TES: ET_A3 mixture intervenes only in the tumor propagation cascades coordinated by IL6, by inhibiting them, the extracts not being active after UV-B irradiation.
- In human melanocyte lesions a positive correlation was demonstrated between tumor progression and MMP-2 expression. Increased MMP-9 expression, on the other hand, was found mainly in the radial growth phase of primary melanoma. Under conditions of UV irradiation, a more pronounced inhibitory effect is observed on MMP 9 and MMP 2, in the case of cells treated with the two mixtures of extracts, attenuating the invasive character of murine melanoma.
- TES: ET_A2 and TES: ET_A3 mixtures provide a concerted effect of reducing tumor spread on the two mechanisms involved in tumor progression (apoptosis and cell cycle sequentiality). Under irradiation conditions, the extracts act only in the case of UV-A, maintaining the trend observed in the basal stage. UV-B radiation is much more aggressive, inducing apoptosis in most cell populations, by generating reactive oxygen species.

Considering the conclusions that emerge from these studies, the complementary biological activity of plant extracts, respectively the interrelation of some tissue recovery mechanisms, they can be exploited and capitalized in authentic cutaneous therapeutic applications, such as:

- **Treatment of wounds of different etiologies:** sage extract (**SvPG**) and glycoalkaloid compounds from tomato waste (**Sol-T**), through their ability to fight inflammatory processes, including those due to microbial infections, and red clover extracts (**ET**) and active components from grape waste (**TES**), by restoring the homeostasis of the protein matrix and cell turnover, with relevance in skin regeneration.
- **Intrinsic and extrinsic photoprotection against environmental pollutants: SvPG** and **Al** biocomplexes through major inhibition of ROS and antiangiogenic and anti-inflammatory effect at the epidermal level.
- **Bacterial skin infections (mycoses, infected wounds): SvPG** and **Sol-T** extracts by the protective action against inflammation and by the anti-irritant effect, manifested by blocking the signaling pathways coordinated by the cytokines IL6, IL8 and IL1α.
- **Stopping effect of hyperproliferative skin disorders (eg melanoma):** the association between bioactive extracts of red clover (**ET**) 74and phytocompounds from grape waste (**TES**) - TES: ET_A2 (ratio 1: 5) and TES: ET_A3 (ratio 1: 3) by reducing melanin (main indicator of malignancy), by anti-inflammatory action in melanocytes subjected to UV-A irradiation, by reducing tumor spread (MMP2 inhibition, induction of apoptosis by generating oxygen reactive species).

BIBLIOGRAPHY

1. Agustin-Salazar S., Medina-Juárez L.A., Soto-Valdez H., F. Manzanares-López F., Gámez-Meza N., Influence of the solvent system on the composition of phenolic substances and antioxidant capacity of extracts of grape (Vitis vinifera L.) marc, Australian Journal of Grape and Wine Research, 20, 208–213 (2014).
2. Albelda S. M., Buck C. A., Integrins and other cell adhesion molecules. The FASEB Journal, 4(11), 2868–2880 (1990).
3. Al-Snafi A.E., The Pharmacological Importance and Chemical Constituents of Arctium Lappa. A Review, IJPRS, 3(1), (2014).
4. Baek J., Lee M-G., Oxidative stress and antioxidant strategies in dermatology, Redox Report, 21:4, 164-169, (2016).
5. Baricevic D., Sosa S., Loggia R.D., Tubaro A., Simonovska B., Krasna A., Zupancic A., Topical anti-inflammatory activity of Salvia officinalis L. leaves: the relevance of ursolic acid, Journal of Ethnopharmacology 75, (2–3) 125–132 (2001).
6. Baroni A., Buommino E., De Gregorio V., Ruocco E., Ruocco V., Wolf R., Structure and function of the epidermis related to barrier properties, Clinics in Dermatology 30, 257–262 (2012).
7. Biswas S.K., Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox?, Oxidative medicine and cellular longevity, 5698931 - 40, (2016).
8. Blois M.S., Antioxidant Determinations by the Use of a Stable Free Radical, Nature, 181, 1199–1200 (1958).
9. Bodemer, A. A.. Psoriasis. Integrative Medicine, 726–738.e2. (2018).
10. Bosch R., Philips N, Suárez-Pérez J.A., Juarranz A., Devmurari A., Chalensouk-Khaosaat J., González S., Mechanisms of photoaging and cutaneous photocarcinogenesis, and photoprotective strategies with phytochemicals, Antioxidants, 4, 248-268 (2015).
11. Boukamp P., Petrussevska R.T., Breitkreutz D., Hornung J., Markham A., Fusenig N.E., Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line, The Journal of Cell Biology, 106, 761-771, (1988).
12. Brenner M., Hearing V.J., The Protective Role of Melanin Against UV Damage in Human Skin, Photochem Photobiol., 84(3): 539–549 (2008).
13. Candrea E., Senila S., Tatomir C., Cosgarea R., Active and inactive forms of matrix metalloproteinases 2 and 9 in cutaneous melanoma, International Journal of Dermatology, 53, 575–580 (2014).
14. Carlo R., Ildo N., Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat. Protoc., 1(3), 1458-1461, (2006).
15. Craig S., Earnshaw C.H., Virós A., Ultraviolet light and melanoma, J Pathol., 244: 578–585 (2018)
16. **Crăciun L.M.**, Dumitriu B.G., Olariu L., Jurcoane S., Cristea S., Adil A, Rosoiu N., Papacocea R., Regenerative and scare healing potential of active compounds from *Camelina sativa* oil and grape pomace. Rom Biotechnol Lett.; 24(6): 1075-1082. (2019) DOI: 10.25083/rbl/24.6/1075.1082.
17. **Crăciun L.M.**, Dumitriu B.G., Olariu L., Ene D.M., Rosoiu N., Antioxidant effect of a vegetal grape waste complex, demonstrated in relevant dermal and epidermal cellular systems, Rom Biotechnol Lett., ISSN: 2248-3942 – în curs de publicare (RBL10850).
18. **Crăciun L.M.**, Dumitriu BG., Ene DM., Abdi A., Olariu L., Rosoiu N., Valorification of Grape Marc by Obtaining BioactiveComplexes Tested Through In Vitro Experimental Models, Academy of Romanian Scientists, Annals Series on Biological Sciences, 2017, ISSN 2285 – 4177.
19. **Craciun M.L.**, Dumitriu B., Ene M.D., Abdi A., Olariu L., Roșoiu N., Valorisation of Vegetable Waste from Solanum sp. and Grapes as Sources of Antioxidant and Antiinflammatory Active Ingredients, AOSR, Autumn Scientific Session, 12-14 sept. 2017.
20. D'Orazio J., Jarrett S., Amaro-Ortiz A., Scott T., UV Radiation and the Skin, Int. J. Mol. Sci, 14, 12222-12248 (2013).
21. Da Silva G.F., Rocha L.W., Meira Quintão N.L., Nutraceuticals, Dietary Supplements, and Functional Foods as Alternatives for the Relief of Neuropathic Pain, Bioactive Nutraceuticals and Dietary Supplements in Neurological and Brain Disease, cap. 10, 87-92 (2015).
22. Dumitriu B., Olariu L., Ene M.D., Zglimbea Z., Rosoiu N., The Photo-Protective Activity of Crataegus monogyna Extract Proved by Complementary Mechanisms of Skin Inflammation, Damage of Extracellular Matrix, Cellular Survival and Proliferation Disorders, International Journal of Biotechnology for Wellness Industries,1, 177-188. (2012).
23. Dumitriu B.G., Ene M.D., Olariu L., Rosoiu N., Craciun L.M., Abdi A, Manda G., Papacocea T., Grape Pomace and Red Clover Extracts Modulate the Proliferative Response of Murine Melanoma Cells, Academy of Romanian Scientists, Annals Series on Biological Sciences, ISSN 2285 – 4177, (2018).
24. El-Bahr S.M., Biochemistry of Free Radicals and Oxidative Stress. Science International, 1, 111-117 (2013).
25. Evers L.H., Bhavsar D., Mailänder P.,The biology of burn injury. Exp Dermatol., 19: 777–783 (2010).
26. Fiedor J., Burda K., Potential Role of Carotenoids as Antioxidants in Human Health and Disease, Nutrients, 6, 466-488 (2014).
27. Figueiredo-González M., Valentão P., Andrade P.B., Tomato plant leaves: From by-products to the management ofenzymes in chronic diseases, Industrial Crops and Products, 94, 621–629 (2016).
28. Fotis L., Agrogiannis G., Vlachos I.S., Pantopoulou A., Margoni A., Kostaki M, Verikokos C., Tzivras D., Mikhailidis D.P., Perrea D., Intercellular Adhesion Molecule (ICAM)-1 and Vascular Cell Adhesion Molecule (VCAM)-1 at the Early Stages of Atherosclerosis in a Rat Model, In Vivo, 26(2):243-50 (2012).
29. Fukai T., Ushio-Fukai M., Superoxide Dismutases: Role in Redox Signaling, Vascular Function, and Diseases, Antioxidants & Redox Signaling, 15(6), 1583-606 (2011).
30. Ghorbani A., Esmaeilizadeh M., Pharmacological properties of Salvia officinalis and its components, J Tradit Complement Med., 7(4): 433–440. (2017).

31. Gragnani A., Cornick S.M., Chominski V., de Noronha S.M.R., de Noronha S.A.A.C., Ferreira L.M., Review of Major Theories of Skin Aging. Advances in Aging Research, 3, 265-284 (2014).
32. He L., He T., Farrar S., Ji L., Liu T., Ma X., Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species, Cell Physiol Biochem., 44, 532-553 (2017).
33. Hoejberg L., Bastholt L., Schmidt H., Interleukin-6 and melanoma, Melanoma Research, 22:327–333 (2012).
34. Huang H-C., Hsieh W-Y., Niu Y-L., Chang T-M., Inhibitory Effects of Adlay Extract on Melanin Production and Cellular Oxygen Stress in B16F10 Melanoma Cells, Int. J. Mol. Sci., 15, 16665-16679 (2014).
35. Huang, T.-H., Lin, C.-F., Alalaiwe, A., Yang, S.-C., & Fang, J.-Y. (2019). Apoptotic or Antiproliferative Activity of Natural Products against Keratinocytes for the Treatment of Psoriasis. International Journal of Molecular Sciences, 20(10), 2558.
36. Hussain T., Tan B., Yin Y., Blachier F., Tossou M., Rahu N., Oxidative Stress and Inflammation: What Polyphenols Can Do for Us?, Oxidative Medicine and Cellular Longevity, 1-9 (2016)
37. Huyut Z., Beydemir S., Gülçin I., Antioxidant and Antiradical Properties of Selected Flavonoids and Phenolic Compounds, Biochem Res Int., 1-10 (2017).
38. Ighodaro O.M., Akinloye O.A., First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid, Alexandria Journal of Medicine, 54, 287–293 (2018).
39. Jamwal K., Bhattacharya S., Puri S., Plant growth regulator mediated consequences of secondary metabolites in medicinal plants, Journal of Applied Research on Medicinal and Aromatic Plants, 9, 26–38 (2018).
40. Kabera J.N., Semana E., Mussa A.R., He X., Plant Secondary Metabolites: Biosynthesis, Classification, Function and Pharmacological Properties, Journal of Pharmacy and Pharmacology, 2, 377-392 (2014).
41. Kammeyer A., Luiten, R.M. Oxidation events and skin aging, Ageing Research Reviews, 21, 16–29. (2015).
42. Kawashima S., Funakoshi T., Sato Y, Saito N., Ohsawa H., Kurita K., Nagata K., Yoshida M., Ishigami A., Protective efect of pre- and postvitamin C treatments on UVBirradiation-induced skin damage, Nature, Scientific Reports, 8:16199, (2018).
43. Kolodziejczyk-Czepas J., Trifolium species – the latest findings on chemical profile, ethnomedicinal use and pharmacological properties, Pharmaceutical Society, Journal of Pharmacy and Pharmacology, 68, 845–861 (2016).
44. Kondo T., Ishida Y., Molecular pathology of wound healing, Forensic Science International, 203, 93–98 (2010).
45. Krueger, G., & Ellis, C. N., Psoriasis—recent advances in understanding its pathogenesis and treatment. Journal of the American Academy of Dermatology, 53(1), S94–S100 (2005).
46. Kruk J., Duchnik E., Oxidative Stress and Skin Diseases: Possible Role of Physical Activity, Asian Pac J Cancer Prev, 15 (2), 561-568 (2014).
47. Krutmann J., Bouloc A., Sore G., Bernard B.A., Passeron T., The skin aging exposome, J Dermatol Sci., 85, 152–161 (2017).
48. Mahecha A.M., Wang H., The influence of vascular endothelial growth factor-A and matrix metalloproteinase-2 and -9 in angiogenesis, metastasis, and prognosis of endometrial cancer, OncoTargets and Therapy, 10 4617–4624 (2017).
49. Malich G., Markovic B., Winder C., The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines, Toxicology, 124(3), 179-92 (1997).
50. Marionnet C., Caroline Tricaud C., Bernerd F., Exposure to Non-Extreme Solar UV Daylight: Spectral Characterization, Effects on Skin and Photoprotection, Int. J. Mol. Sci., 16, 68-90 (2015).
51. Matejuk A. Skin Immunity, Arch. Immunol. Ther. Exp., 66, 45–54 (2018).
52. Molyneux P., The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity Songklanakarin J. Sci. Technol., 26(2) : 211-219 (2004).
53. Naidoo K., Birch-Machin M.A., Oxidative Stress and Ageing: The Influence of Environmental Pollution, Sunlight and Diet on Skin, Cosmetics, 4(4), 1-8 (2017).
54. Norman M. U., James W. G., Hickey M. J. Differential roles of ICAM-1 and VCAM-1 in leukocyte-endothelial cell interactions in skin and brain of MRL/fas lpr mice. Journal of Leukocyte Biology, 84(1), 68–76. (2008).
55. Oliveira I., a, Sousa A., Valentao P., b, Andrade P.B. b, Ferreira I., Ferreres F., Bento A., Seabra R., Estevinho L., Pereira J.A., Hazel (Corylus avellana L.) leaves as source of antimicrobial and antioxidative compounds, Food Chemistry, 105, 1018–1025 (2007).
56. Oliveira I., Sousa A., Morais J., Ferreira I., Bento A., Estevinho L., Pereira J.A., Chemical composition, and antioxidant and antimicrobial activities of three hazelnut (Corylus avellana L.) cultivars, Food and Chemical Toxicology 46 ,1801–1807 (2008).
57. Oroian, M.; Escriche R., Antioxidants: Characterization, natural sources, extraction and analysis. Food Research International, 74,10-36 (2015).
58. Pehlivan F.E., Vitamin C: An Antioxidant Agent. cap 2 in Vitamin C, Amal H. Hamza, IntechOpen, (2017).
59. Phaniendra A.,Jestadi D.B., Periyasamy L., Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases, Ind J Clin Biochem., 30(1):11–26 (2015).
60. Pisoschi A.M., Pop A., The role of antioxidants in the chemistry of oxidative stress: a Review, Eur J Med Chem., 97, 55-74 (2015).
61. Pittayapruek P., Meephansan J., Prapapan O., Komine M., Ohtsuki M., Role of Matrix Metalloproteinases in Photoaging and Photocarcinogenesis, Int. J. Mol. Sci., 17, 868 (2016).
62. Pogačnik L., Poklar Ulrih N., Application of optimized chemiluminescence assay for determination of the antioxidant capacity of herbal extracts Luminescence, 1-6 (2012).

63. Predes F.S., Ruiz A., CarvalhoJ.E., Foglio M.A., Dolder H., Antioxidative and in vitro antiproliferative activity of Arctium lappa root extracts, Complementary and Alternative Medicine, 11(25), 1472-6882 (2011).
64. Prieto-Granada N.C., Lobo A., Mihm C.M., 20 - Skin Infections, Diagnostic Pathology of Infectious Disease (Second Edition), 542-647 (2018).
65. Reichrath J, Rass K Ultraviolet damage, DNA repair and vitamin D in nonmelanoma skin cancer and in malignant melanoma: an update. Adv Exp Med Biol 810: 208-233(2014).
66. Reuter S, Gupta S.C, Chaturvedi M.M.,. Aggarwal B.B, Oxidative stress, inflammation, and cancer: how are they linked? Free Radic Biol Med., 49(11): 1603–1616) (2010).
67. Rinnerthaler M., Bischof J., Streubel M.K., Trost A., Richter K., Oxidative Stress in Aging Human Skin, Biomolecules, 5, 545-589 (2015).
68. Riss T.L., Moravec R.A., Niles A.L., Cell Viability Assays. [Updated 2016]. In: Sittampalam GS, Coussens NP, Brimacombe K, et al., editors. Assay Guidance Manual (2013).
69. Robert A., Lawley J.S., Lawley J.T, Role of Microvascular Endothelial Cells inInflammation, J Invest Dermatol, 100:111S–115S, (1993).
70. Robinson J.P., Current protocols in Cytometry, 9.7.1 – 9.7.14 (1997).
71. Rungratanawanich W., Memo M., Uberti D., Redox Homeostasis and Natural Dietary Compounds: Focusing on Antioxidants of Rice (Oryza sativa L.), Nutrients, 10, 1605, 1-19 (2018).
72. Saba E., Oh M-J., Kwak D., Roh S-S., Kwon H-W., Kim S-D., Rhee M.H., Solanum lycopersicum (tomato) ethanol extract elicits anti-inflammatory effects via the nuclear factor kappa B pathway and rescues mice from septic shock, Korean J Vet Res, 57(2) : 97-104 (2017).
73. Sazdanić D., Mikulić M., Kladar N., Hogervorst J., Atanacković Krstonošić M., Analysis of the factors influencing red clover (Trifolium pratense L., Fabaceae) isoflavone content, Biologia Serbica, 40(2), 34-41 (2018) DOI 10.5281/zenodo.2452290
74. Siddique M.A.B., Brunton N., Food Glycoalkaloids: Distribution, Structure, Cytotoxicity, Extraction, and Biological Activity, Alkaloids - Their Importance in Nature and Human Life, Joanna Kurek, IntechOpen, (2019).
75. Sies H., On the history of oxidative stress: Concept and some aspects of current development Current Opinion in Toxicology, 7, 122–126 (2018).
76. Silva-Beltrán N.P., Ruiz-Cruz S., Cira-Chávez L.A., Estrada-Alvarado M.I., Ornelas-Paz J., López-Mata M.A., Del-Toro-Sánchez C.L., Ayala-Zavala J.F., Márquez-Ríos E., Total Phenolic, Flavonoid, Tomatine, and Tomatidine Contents and Antioxidant and Antimicrobial Activities of Extracts of Tomato Plant, International Journal of Analytical Chemistry, 4, 1-10, (2015).
77. Stojiljković D., Pavlović D., Arsić I., Oxidative Stress, Skin Aging and Antioxidant Therapy, Scientific Journal of the Faculty of Medicine in Niš, 31(4):207-217(2014).
78. Thomsen S.F., Atopic Dermatitis: Natural History, Diagnosis and Treatment, ISRN Allergy, 1-7 (2014).
79. Toshikazu K., Yuko I., Molecular pathology of wound healing, Forensic Science International 203, 93–98 (2010).
80. Trouba K.J., Hamadeh H.K., Amin R.P., Germolec D.R., Oxidative Stress and Its Role in Skin Disease, Antioxid. Redox Signal. 4, 665–673 (2002).
81. Tsala D.E., Amadou D., Habtemariam S., Natural wound healing and bioactive natural products Phytopharmacology, 4(3), 532-560 (2013).
82. Velickovic D.T., Randjelovic N., Ristic M.S., Velickovic A., Smelcerovic A., Chemical constituents and antimicrobial activity of the ethanol extracts obtained from the flower, leaf and stem of Salvia officinalis L., J.Serb.Chem.Soc. 68(1), 17–24 (2003).
83. Verdier-Se´vrain S., Effect of estrogens on skin aging and the potential role of selective estrogen receptor modulators, Climacteric, 10:289–297 (2007).
84. Vila R., Blanca Freixa B., Cañigueral S., Antifungal compounds from plants, Recent Advances in Pharmaceutical Sciences III,: 23-43 (2013).
85. Wang P.-H., Huang B.-S., Horng H.-C., Yeh C.-C., Chen Y.-J., Wound healing, J Chin Med Assoc., 81, 94-101 (2018).
86. Wang Y., Branicky R., Noë A., Hekimi S., Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling, J. Cell Biol. 217(6), 1915-1928 (2018).
87. Waringa W.S., Mishrab V., Maxwell S., Comparison of spectrophotometric and enhanced chemiluminescent assays of serum antioxidant capacity, Clinica Chimica Acta, 338, 67–71 (2003).
88. Weydert C.J., Cullen J.J., Measurement of superoxide dismutase, catalase, and glutathione peroxidase in cultured cells and tissue, Nat Protoc., 5(1), 51–66 (2010).
89. Wlaschek M., Tantcheva-Poor I., Naderi L., Ma W., Schneider L.A., Razi-Wolf Z., Schuller J., Scharffetter-Kochanek K., Solar UV irradiation and dermal photoaging, J Photochem Photobiol B, 63, 41–51 (2001).
90. Xu H., Zheng Y.-W., Liu Q., Liu L.-P., Luo F.-L., Zhou H.-C., Li Y.-M., Reactive Oxygen Species in Skin Repair, Regeneration, Aging, and Inflammation. Reactive Oxygen Species (ROS) in Living Cells, cap. 5, 69-87 (2018).
91. Xu Y., Fisher G. J., Ultraviolet (UV) light irradiation induced signal transduction in skin photoaging. Journal of Dermatological Science Supplement, 1(2), S1–S8 (2005).
92. Xue M., Le N., Christopher J Jackson C..J., Targeting matrix metalloproteases to improve cutaneous wound healing, Expert Opin. Ther. Targets 10(1):143-155 (2006).

PUBLICATIONS

A. Scientific papers published in ISI journals:

1. **Crăciun M.L.**, Dumitriu B.G., Olariu L., Jurcoane S., Cristea S., Adil A., Rosoiu N., Papacocea R., Regenerative and scare healing potential of active compounds from Camelina sativa oil and grape pomace, **Rom Biotechnol Lett**., ISSN: 2248-3942, 2019/ **Impact Factor (ISI) – 0.59/2018.** DOI: 10.25083/rbl/24.6/1075.1082, https://www.e-repository.org/rbl/vol.24/iss.6/20.pdf
2. Olariu L., Dumitriu B.G., **Craciun M.L.**, Buse E., Rosoiu N., Bojinca M., Papacocea T.,The in vitro influence of a pharmaceutically active small sea fish extract on apoptosis and proliferation mechanisms amplified by inflamatory conditions, **Farmacia**, 2018, **Impact Factor - 1.527/2018** http://www.revistafarmacia.ro/201803/2018-03-art-19-Olariu Bojinca Papacocea 524-529.pdf
3. **Crăciun M.L.**, Dumitriu B.G., Olariu L., Ene D.M., Abdil A., Rosoiu N., Antioxidant effect of a vegetal grape waste complex, demonstrated in relevant dermal and epidermal cellular systems, **Rom Biotechnol Lett**., ISSN: 2248-3942 **Impact Factor (ISI) – 0.59/2019** – în curs de publicare (RBL10850)
4. Paulet M., Ciobica A., Olariu L., Ene M.D., Antioch I., Ababei D., **Craciun M.L.**, Adil A., Rosoiu N., Some preliminary results regarding the effects of grape pomace on memory and anxiety in mice, **Rom Biotechnol Lett.,** ISSN: 2248-3942 **Impact Factor (ISI) – 0.59/2019** – în curs de publicare (RBL-2019-0036)
5. Draga-Coleta S.V., Olariu, L., Dumitriu B.G., Ene M.D., **Craciun M.L.**, Apoptotic and antiproliferative processes from dysplastic and metastatic prostate cells, modulated by proteolytic enzymes of entomological origin, P.09-224, FEBS Open Bio 8 (Suppl.S1) (2018) 105–106, DOI: 10.1002/2211-5463.12453. **Impact Factor (ISI) – 4.73/2018** https://febs.onlinelibrary.wiley.com/doi/epdf/10.1002/2211-5463.12453
6. **Craciun M.L.**, Ene M.D., Buse E., Pyatigorskaya N., Pavlov A., Olariu L., Cellular and molecular effects of a small sea fish extract on hyaluronan homeostasis, The FEBS Journal 284 (Suppl. 1), pag. 382, (2017). **Impact Factor (ISI) – 4.53/2017**
7. **Craciun M.L.**, L. Olariu, B. Dumitriu, N. Rosoiu, Molecular mechanisms involved in inflammation modulated by vegetal active principles, P-04.04.4-010 The FEBS Journal 283 (Suppl. 1) (2016) 129–417 DOI: 10.1111/febs.13808, pg.355, **Impact Factor (ISI) – 3.90/2016**
8. Ene D.M., Pyatigorskaya N., Pavlov A., Dumitriu B., **Craciun M.L.**, Olariu L., Cellular oxidative processes relevant for articular degenerative pathologies modulated by an active extract from small sea fish, P-09.04.4-011 The FEBS Journal 283 (Suppl. 1) (2016) 129–41 7 DOI: 10.1111/febs.13808, pg.385, **Impact Factor (ISI) – 3.90/2016**
9. **Craciun M.L.**, Olariu L., Ene MD., Zglimbea L., In vitro modulation of enzymatic processes using biologic active substances with relevance for cancer therapy, FEBS Journal 281 (Suppl. 1) (2014) 65–783 Issn Print: 1742-464X, **Impact Factor (ISI) – 4.00/2014**

B. Scientific papers published in extenso in BDI journals:

1. **Craciun M.L.**, Dumitriu BG, Rosoiu N, Ene MD, Manda G, Olariu L, Entomological Compounds Impact on Key Factors of Prostate Adenocarcinoma Progression, Academy of Romanian Scientists Annals - Series on Biological Sciences, 2019, ISSN 2285 – 4177 http://www.aos.ro/wp-content/anale/BVol8Nr1Art.4.pdf
2. Dumitriu B.G., Ene M.D., Olariu L., Rosoiu N., **Craciun M.L.**, Abdi A, Manda G., Papacocea T., Grape Pomace and Red Clover Extracts Modulate the Proliferative Response of Murine Melanoma Cells, Academy of Romanian Scientists, Annals Series on Biological Sciences, ISSN 2285 – 4177, 2018 http://www.aos.ro/wp-content/anale/BVol7Nr2Art.9.pdf
3. **Crăciun M. L.**, Dumitriu B.G., Ene D.M., Abdi A., Olariu L., Rosoiu N., Valorification of Grape Marc by Obtaining BioactiveComplexes Tested Through In Vitro Experimental Models, Academy of Romanian Scientists, Annals Series on Biological Sciences, 2017, ISSN 2285 – 4177 http://www.aos.ro/wp-content/anale/BVol6Nr2Art.1.pdf
4. **Crăciun M.L.**, Vacaru A.M., Ene M.D., Vacaru A.M., Olariu, L., Roşoiu, N., pH influence on different bovine testicular hyaluronidase determination assays, Academy of Romanian Scientists, Annals Series on Biological Sciences, 2016. ISSN 2285 – 4177 http://aos.ro/wp-content/anale/BVol5Nr1Art.9.pdf

C. Scientific papers published in summary - in BDI journals:

1. Olariu L., Craciun M.L., Ene M.D., Dumitriu B., Serbu S., Nita R., New approaches in prostate therapy through entomological compounds with biological activity at cellular and molecular level, Catalogul Salonului PRO-INVENT 2020 – în curs de publicare
2. Serbu S., Jurcoane S., Dumitriu B., Olariu L., **Craciun M.L.**, Cristea S, Copaci S., Rosoiu N., Dermatocosmetic innovations in skin regeneration and antioxidant protection against environmental pollutants, Catalogul Salonului PRO-INVENT 2020 – în curs de publicare
3. Olariu L., Dumitriu B., Ene M.D., **Craciun M.L.**, Bicu A., Innovative association between entomological compounds and plant extracts in order to design pharmaceutically active complexes, Iasi, Mai 2019, Euroinvent, (**medalie de aur**).
4. Abdi A., **Craciun M.L.**, Dumitriu B., Olariu L., *In vitro* biological action synergisms in proliferative cutaneous processes with applicability in bioproduction development, National Scientific Conference, Academy of Romanian Scientists, Book of abstracts, Volume 13 issue 1, 2019.
5. **Craciun M.L.**, Dumitriu B., Rosoiu N., Ene M.D., Olariu L., Entomological Active Principles with Antitumor Properties on Prostate Adenocarcinoma Cell Line DU 145, National Scientific Conference, Academy of Romanian Scientists, Book of abstracts, Volume 13 issue 1, 2019.

6. Dumitriu B.G., Ene M.D., Olariu L., Rosoiu N., **Craciun M.L.**, Abdi A., Manda G., Papacocea T., Grape Pomace and Red Clover Extracts Modulate the Proliferative Response of Murine Melanoma Cells, National Scientific conference Academy of Romanian Scientists, Scientific research for Sustainable Development, book of abstracts, volume 12, issue 1, 2018.
7. Paulet M., Ciobica A., Olariu L., Ene M.D., Antioch I., Ababei D., **Craciun M. L.**, Adil Abdi, Rosoiu N., Some Preliminary results regarding the effects of grape pomace on memory and anxiety in mice, National Scienticonference Academy Of Romanian Scientists, Scientific research for Sustainable Development, book of abstracts, volume 12, issue 1, 2018.
8. Olariu L., Buse E., Dumitriu B., **Craciun M.L.**, Draga-Coleta S.V., Papacocea R., New cellular and molecular effects, relevant for osteoarthicular pathology, proved for and Romanian original product on specific, predictive, in vitro systems, Iasi, 19 mai 2018, EUROINVENT, (**medalie de aur**).
9. Olariu L., Dumitriu B., Ciuhrii V., **Crăciun M.L.**, Roșoiu N., Entomological Complex with Pro-Apoptotic and Antiproliferative Effect on Prostatic Dysplasia Cells, AOSR , Autumn Scientific Session, 12-14 sept. 2017.
10. **Craciun M.L.**, Ene M.D., Olariu L., Rosoiu N., Study on enzymatic activity of hyaluronidase from bovine testes using different substrate sources, AOSR , Spring Scientific Session, mart. 2017.
11. **Craciun M.L.**, Dumitriu B., Ene M.D., Abdi A., Olariu L., Roșoiu N., Valorisation of Vegetable Waste from Solanum sp. and Grapes as Sources of Antioxidant and Antiinflammatory Active Ingredients, AOSR, Autumn Scientific Session, 12-14 sept. 2017.
12. Olariu L., Vacaru A., Pyatigorskaya N., Vacaru A.M., Pavlov A., Dumitriu B.G., Ene M.D., **Craciun M.L.**,Original romanian product involved in cellular chondro - modulatory mechanisms, EUROINVENT 2016, Catalog EUROINVENT pg. 437. (**medalia de aur**).
13. Olariu L., Dumitriu B.G., **Crăciun M. L.**, Roşoiu N., Cascade proinflamatorii modulate citokinic de extracte bioactive de Salvia officinalis, Asculum hippocastanum şi Calendula officinalis,– prezentare orala la Sesiunea Ştiinţifică de Toamnă a AOŞR, septembrie 2016, Durău – Neamț.
14. Olariu L., Dumitriu B., **Craciun M.L.**, Ene M.D., Rosoiu N., Extract de peste marin marunt- agent antioxidant activ in procese celulare associate cu patologii degenerative articulare, – prezentare orala la Sesiunea Ştiinţifică de Toamnă a AOŞR, septembrie 2016, Durău – Neamt.
15. **Crăciun, M.L.,** Vacaru, A.M., Ene, M.D., Vacaru, A.M., Olariu, L., Roşoiu, N., pH influence on different bovine testicular hyaluronidase determination assays, Conferinta AOSR, Martie 2016, Bucuresti, Romania.

D. Participation in international scientific congresses

1. 39th FEBS–EMBO Conference,2014, Paris, France – poster
2. 41st FEBS Congress, Molecular and Systems Biology for a Better Life, 2015 was scheduled for Kuşadası (live event cancelled), Turkey – abstract
3. 42nd FEBS Congress, From Molecules to Cells and Back, 2017,Jerusalem, Israel – poster
4. 43rd FEBS Congress, Biochemistry Forever, 2018, Prague, Czech Republic
5. European Exhibition of Creativity and Innovation, 2016, Iași, România - poster
6. European Exhibition of Creativity and Innovation, 2018, Iași, România – poster

E. Participation as a team member in national / international research projects:

1. **UEFISCDI/INOVARE/CTR 3 SEH/2012**

Impact eficient pe piata externa al unui produs romanesc high-tech inovativ in algoritmul de restabilire a homeostaziei in boli inflamatorii, degenerative si reumatice.

2. **UEFISCDI/INOVARE/CTR 26DPST/2012**

Valorificarea unor compuşi entomologici eficienţi în diverse patologii degenerative - capacitate inovativă autentică a unui IMM românesc pe piaţa farmaceutică.

3. **POSCCE/ ID 1639/SMIS 47515 /546/29.08.2013**

Solutii inovative pentru determinarea gradului de contaminare cu reziduuri toxice-metale grele si pesticide-in produse de origine vegetala

4. **PN-III-P2-2.1-PTE-2016-0160/ CTR 49**

Prototip de produse medicamentoase cu eliberare controlata pe baza de compozite chitosan-zeolit;

5. **PN-III-P2-2.1-PTE-2016-0166/ CTR 21**

Prototipuri pentru un biopesticid si un produs antimicotic pe baza de biocomplexe vegetale obtinute prin biotehnologii superioare de exploatare a speciilor de Solanum si Camelina sativa.

6.Proiect AOSR, 2017-2019

Valorificarea reziduurilor din vinificatie ca aditivi alimentari si antioxidanti in industrie