Academy of Romanian Scientists *Volume 8, No. 2, 2019, pp. 5 - 16*
Annals Series on Biological Sciences ISSN ONLINE 2285 – 4177

ORIGINAL PAPERS

Oxidative Stress - Trigger of Cutaneous Degenerative Processes; Modulation with Active Principles from Aesculus Hippocastanum, Calendula Officinalis and Vitis Vinifera

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Abstract

The endogenous contribution of natural antioxidants corrects the redox imbalance by mediating the evolution of the disorders caused and/ or stimulated by the oxidative stress. The plant extracts contain natural antioxidants with a role in radical detoxification, constituting valuable resources in the pharmaceutical and cosmetic industry. Compounds such as alkaloids, flavonoids, tannins, terpenoids, saponins, phenolic derivatives, are involved in different phases of the skin healing process through modulation of anti-inflammatory, immunomodulatory, antiviral, or tissue remodeling mechanisms. Our research was carried out in order to highlight a cumulative antioxidant action, on multiple cellular pathways, for the compounds of Calendula officinalis (marigold) -Gb and Aesculus hippocastanum (chestnut) -C in combination with the extract of grape marc (Vitis vinifera) - TES. The study of two relevant cell lines, HaCaT (normal human keratinocytes) and HS27 (human dermal fibroblasts), was considered. Cells were differentially stimulated for in vitro modeling of proinflammatory conditions representative of bacterial invasion (with LPS - bacterial lipopolysaccharide) and pro-oxidative inflammation (PMA- forbolmiristat acetate) respectively. It was highlighted the role of plant extracts in maintaining cellular resistance against oxidative stress interfering in enzymatic cascades and counteracting the attack of oxygenated free radicals (elimination of O2- anions and H2O2 decomposition). The results also confirm the synergism of action of the active compounds tested, directing the researches towards the development of topical use product with skin regenerative effect.

Keywords*:* antioxidant, grape marc, *Calendula off., Aesculus Hipopocastanum, Vitis Vinifera*

Introduction

Skin tissue contains enzymatic or non-enzymatic antioxidant defense systems, with regulatory role of ROS levels, thus maintaining physiological homeostasis [1]. ROS generation in the skin naturally occurs as part of normal cellular metabolism, such as mitochondrial respiration, enzymatic activity or under the action of UV radiation. Maintaining redox homeostasis is essential for the preservation of physiological functions as ROS/ RNS are generated continuously in normal cell metabolism [2]. Antioxidants can exert their effects through various mechanisms, such as suppressing the formation of active species by reducing hydroperoxides (ROO•) and H_2O_2 and also by immobilizing metal ions, capturing active free radicals, repairing and/ or damaging lesions [3]. Similarly, some antioxidants also induce biosynthesis of other antioxidants or defense enzymes. The bioactivity of an antioxidant is dependent on several factors, including their structure, physico-chemical characteristics and its capacity to generate radicals *in vivo*. [4].

Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus maintaining systemic health. However, under conditions that promote oxidative stress, endogenous antioxidants may not be sufficient, and dietary antioxidant intake is required to maintain optimal cellular functions. Enzyme antioxidant systems involve superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GSH-Px). (O_2) is transformed in the presence of SOD into H_2O_2, which is decomposed into water and oxygen by CAT, preventing the production of hydroxyl radicals. In addition, GSH-Px converts peroxides and hydroxyl radicals into nontoxic forms by oxidation of reduced glutathione (GSH) to glutathione disulfide and then reduced to GSH by glutathione reductase [1, 3].

The pathogenicity of skin disorders involves complex phenomena of physiological, immunological, genetic nature, etc., generated by two main agents (oxidants and cytokine network), which are involved in various skin disorders (such as psoriasis, atopic dermatitis, contact dermatitis, virtiligo, etc), including carcinogenesis, lesions caused by UV radiation, inflammatory processes [5, 6]. Oxidative stress plays a key role in the pathogenesis of psoriasis, manifested by the increased generation of ROS by leukocytes infiltrating psoriatic lesions. In the blood of patients with psoriasis, low levels of antioxidants have been identified along with an increase in lipid peroxidation markers, as well as enhancement of serum catalase activity and superoxide dismutase [7, 8]. Natural compounds can ameliorate psoriatic lesions through some molecular mechanisms related to apoptosis, angiogenesis inhibition, inflammation suppression [9, 10, 11]. Both contact dermatitis and atopic dermatitis have similar clinical, histological and molecular characteristics, but have different pathogenesis. Atopic dermatitis is a chronic, inflammatory skin condition that most often starts in childhood, and is

followed in some cases by other diseases (allergic rhinitis, allergic asthma, etc.)[12].

Another chronic skin condition, virtiligo, is a depigmenting disease characterized by progressive degradation of epidermal melanocytes, which is associated with a polymorphism of the genes involved in the immune response and melanogenesis and arises as a result of complex interactions between biochemical and immunological processes. In the active phase of the disease, ROS and oxygenated water are generated excessively, causing disruption of biological processes, thus an excess of H_2O_2 has been shown to affect the activity of tyrosinase (a key enzyme in melanogenesis) by oxidizing methionine residues, but also other proteins and peptides with relevant role in oxidative processes [13].

Herbal extracts are increasingly used in medicine and are important sources of natural antioxidants that enhance endogenous antioxidant defense against lesions caused by reactive species and restore optimal balance by neutralizing them [14]. Antioxidant systems are classified into two major groups, protective or enzymatic antioxidants and non-enzymatic antioxidants. The main classes of compounds with antioxidant activity are: vitamins, carotenoids and polyphenols (flavonoids, flavonols, flavones, flavanones, anthocyanins, isoflavones, phenolic acids, tannins, stilbenes and lignans) [15]. Antioxidant defense mechanisms of the human body are not always able to combat the attack of free radicals, which is why it is necessary to provide compounds with scavenger properties, the plants being used more and more as natural ingredients in skin protection products [16]. Based on the phytochemical composition and biomedical applications of the extracts of *Aesculus hippocastanum* (used in the treatment of chronic venous insufficiency, hemorrhoids, local ulcers, cancer, etc. [17, 18]) and *Calendula officinalis* (treatment of skin ulcers, eczema, conjunctivitis, acne, etc. [19-22]), their association with a grape marc extract from the processing of vinification wastes (with antioxidant action, modulators of cell proliferation, in the process of keratinocyte differentiation, matrix restructuring demonstrated in our previous studies [23-26]), leads to the enhancement of their biological activity and the enlarging of the spectrum of action. Our studies aim to point out the efficacy of some original associations of antioxidant extracts (*Calendula officinalis, Aesculus hippocastanum, grape mark extract)* on regenerative mechanisms at dermal and epidermal level.

Material and methods

- **Chemicals**

Standardized cell lines - normal human fibroblasts (HS27) and normal immortalized human keratinocytes (HaCaT) from ATCC. Cell lines were cultured according to ATTC protocols (37°C, in a humid atmosphere containing 5% CO_2) and once they reach the confluence they are distributed in plates for adhesion to

the substrate for 24 hours, subsequently being treated with the substances of interest for 48 hours under the same growing conditions.

Reagents:

DMEM culture medium (Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 Ham), bovine fetal serum and antibiotic solution (containing 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per mL), were purchased from Sigma Aldrich. The keratinocytes culture medium Dulbecco's Modified Eagle's Medium (DMEM) was purchased from ATCC®. Oxygenated water, potassium phosphate, N, N, N ′, N′-ethylenediaminetetraacetic acid (EDTA), Cytochrome c, Xanthine, Xanthine oxidase (XOD), Superoxide dismutase (SOD), Hank`s Buffer Salt Solution (HBSS), 2', 7'-dichlorfluorescein diacetate (DCFH-DA), dihydroethidine (HE), phorbolmiristat acetate and lipopolysaccharide, N-acetyl-cysteine (NAC) were purchased from Sigma-Aldrich. Mouse F (ab) 2 IgG (H + L) PE-conjugated Antibody was purchased from R&D Systems, Anti-Glutathione antibody [D8] and Mouse IgG2c, monoclonal kappa [18C8BC7AD10] - Isotype Control, was purchased from ABCAM.

- **Preparation of the bioactive extracts**

The plant extracts: TES (grape marc extract), Gb (*Calendula officinalis* extract) and Cs (*Aesculus hippocastanum* extract), obtained by own technological processes, were associated in different proportions (v/v), as presented in the Table 1.

Table 1) Association of TES, Gb and Cs active biocomplexes

	A1	**B1**	**C1**	**A2**	**B2**	**C2**
TES	1	1	9	1	1	9
Gb	9	1	1			
Cs				9	1	1

1.1. Evaluation of cellular glutathione (GSH) by flow cytometry

Glutathione has a dual role: as an antioxidant by keeping the –SH groups of proteins in reduced form and in the detoxification processes, binding heavy metals, solvents, pesticides. Its detection is important in estimating the intrinsic antioxidant cell status. Determination of intracellular GSH will be done by labeling with fluorescent antibodies (anti-glutathione ABCAM antibody; secondary antibody - Alexa-Fluor 488 conjugated goat anti-mouse IgG (H + L) and analysis by flow cytometry. Fluorescence emission is compared with Mouse IgG2a Monoclonal Isotype Control (AB10191- ABCAM.).

1.2. Simultaneous identification of intracellular oxygenated radicals (superoxide anion and oxygenated water) by flow cytometry

The method uses 2', 7'-dichlorofluorescein diacetate (DCFH-DA) to measure the production of oxygenated water, which oxidizes the nonfluorescent sample to a fluorescent one that is detected by flow cytometry. After cell activation, NADPH oxidase catalyzes the reduction of molecular oxygen (O_2) to superoxide anion (O_2^-)

which is then reduced to oxygenated water. H_2O_2 together with the peroxidases have the ability to oxidize DCFH captured to DCF, a strongly fluorescent compound at 530nm. The production of green fluorescence is proportional to the amount of oxygenated water generated. Hydroxyethidine (HE) is used for the determination of superoxide anion and can be used both alone and in combination with DCFH-DA. HE readily penetrates into the cell membrane, then is oxidized to ethidium bromide, which is captured in the nucleus by interleaving the DNA chain and emitting at 620nm (PE_A) [27].

1.3. Determination of enzymatic activity of catalase

The activity of catalase in the cell samples was determined according to the protocol described by Aeby H [28] with minor modifications, following spectrophotometrically the decrease of the absorbance of the samples to $\lambda = 240$nm, as a result of the decomposition under the action of catalase of H_2O_2 in water and oxygen. The difference in absorbance (DA_{240}) per unit time is a measure of the activity of catalase. One unit will decompose 1.0 μmol of H_2O_2 / minute at pH 7.0 and 25 ° C.

1.4. Determination of enzymatic activity of superoxide dismutase (SOD)

To determine the enzymatic activity of superoxide dismutase from cell lysates, the working protocol was applied from Sigma Aldrich, as follows: a reaction cocktail of 23ml purified water, 25ml phosphate buffer, 1ml EDTA solution, 1ml cytochrome c and 50ml xanthine solution was prepared, with pH adjustment at 7.8 at 25°C. The control (reaction cocktail and purified water), a sample without inhibitor (reaction cocktail and xanthine oxidase solution) and a sample containing additional inhibitor to be tested (superoxide dismutase from cell lysate or bovine source) are prepared. The absorbance is read at 550nm at the beginning of the reaction and the absorbance change (ΔA) is monitored for 5min. [29].

Results and discussions

The assessment of intracellular oxidative stress was performed by flow cytometry analysis methods, monitoring reactive oxygen species (hydrogen peroxide and superoxide anion - DCFH-DA, respectively HE) and quantifying intracellular glutathione. The determination of the antioxidant impact of the extracts was evaluated through the enzymatic activity of two phase I oxidative enzymes, respectively catalase (reaction of decomposition of oxygenated water in water and O_2) and superoxide dismutase (monitoring the inhibition of the process of reduction of Cytochrome c by the superoxide radical).

1.5. Evaluation of intracellular GSH content using flow cytometry.

As we previously presented, the antioxidant status had major changes in skin inflammatory disturbances, and it is important to stimulate the intrinsic potential of relief, as well as to change the balance of reactive oxygen species.

In order to mimics the pro-inflammatory aggression related to different pathological conditions, we design a study with the following stimulation conditions: bacterial inflammation accompanied by oxidative stress trigger - LPS + PMA

(1µg/ml + 0.1µM), 18 hours of stimulation (HS27, HaCaT); bacterial inflammation: LPS, a lipopolysaccharide extracted from the Escherichia coli bacteria - 1µg/ml 18 hours of stimulation (HaCaT); endogenous oxidative stimulation: PMA (Phorbol 12-myristate 13-acetate) 1µM for 4h (HaCaT). The positive control used was the antioxidant compound - N-acetyl-cysteine (NAC). The results are presented in the graphs below as a percentage variation from the corresponding control – Fig.1 and 2.

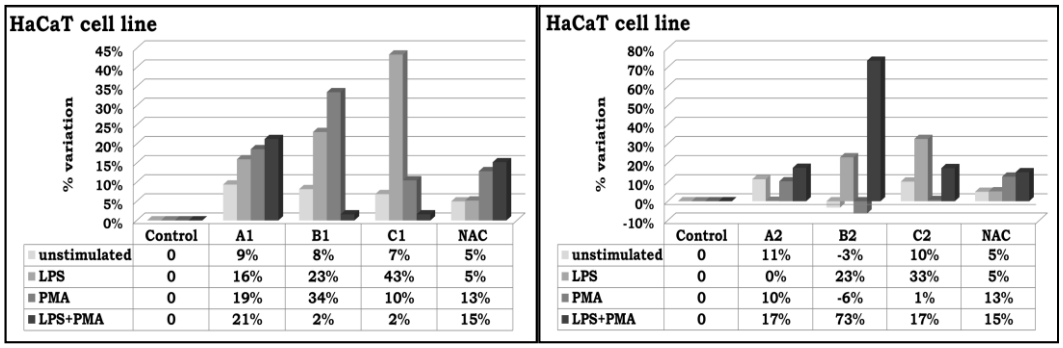


Fig. 1. The effect of the associated **TES**, **Gb** and **Cs** extracts on intracellular GSH level in human keratinocytes:

a. Keratinocytes treated with0.1% **TES:Gb** combinations in proportions of 1:9 (A1), 1:1 (B1) and 9:1 (C1).

b. Keratinocytes treated with 0.1% **TES**:**Cs** combinations in proportions of 1:9 (A2), 1:1 (B2) and 9:1 (C2)

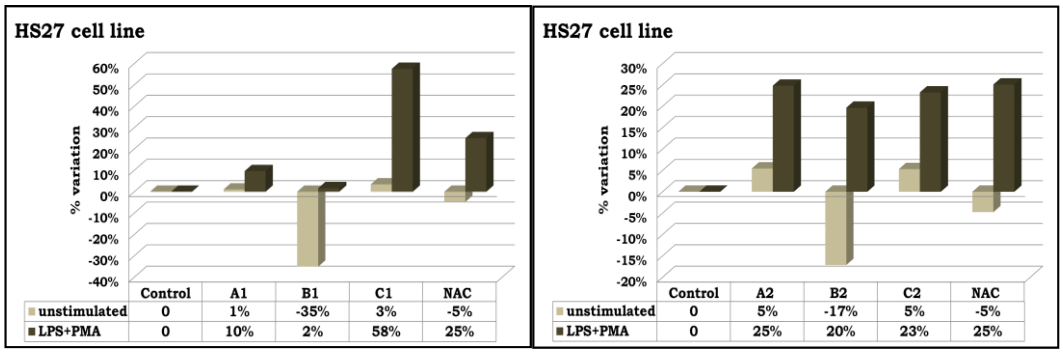


Fig. 2. Intracellular GSH level in human fibroblasts treated with **TES**, **Gb** and **Cs** extracts combinations in stimulated conditions (LPS+PMA):

a. Fibroblasts treated with 0.1% **TES**:**Gb** combinations in proportions of 1:9 (A1), 1:1 (B1) and 9:1 (C1).

b. Fibroblasts treated with 0.1% **TES**:**Cs** combinations in proportions of 1:9 (A2), 1:1 (B2) and 9:1 (C2)

Considering the general antioxidant effects, expressed on every inflammatory path we tested, the most significant effects on intracellular glutathione stimulation was noticed for the following active principles combination: A1 (TES: Gb =1: 9), B1 (TES: Gb =1:1), C1 (TES: Gb = 9:1) and

C2 (TES: Cs =9:1). Mixtures A2 ((TES: Cs =1:9) and B2 (TES: Cs =1:1) increase the intracellular glutathione only after stimulation with LPS + PMA, being effective in the control of intrinsic oxidative stress produced by inflammation associated with bacterial infections. As well as, at the dermal fibroblasts level, A2 (↗25%), B2 (↗20%) and C1 (↗58%), exhibit a strong intrinsic antioxidant effect, rising the intracellular glutathione.

1.6. Extrinsic antioxidant effect by intracellular modulation of oxidative balance in the oxygenated free radicals - oxidative enzymes (catalase and superoxide-dismutase) system.

The enzymatic activity of the main antioxidant enzymes: catalase and superoxide-dismutase was evaluated from normal human keratinocytes lysates, cultured as follows: 24h adhesion, 48h treatment under normal development conditions (basal level of hydrogen peroxide, in the presence and absence of the investigated compounds), as well as within a nonspecific oxidative stimulation model induced by the treatment with LPS and PMA (24h pretreatment with extract + 24h stimulation). The results are shown in the graphs below, as enzyme concentration in the treated samples and the control **– Table 2**.

Table 2. Modulation of enzymatic activity of catalase and superoxide dismutase in HaCaT cells by bioactive extracts tested

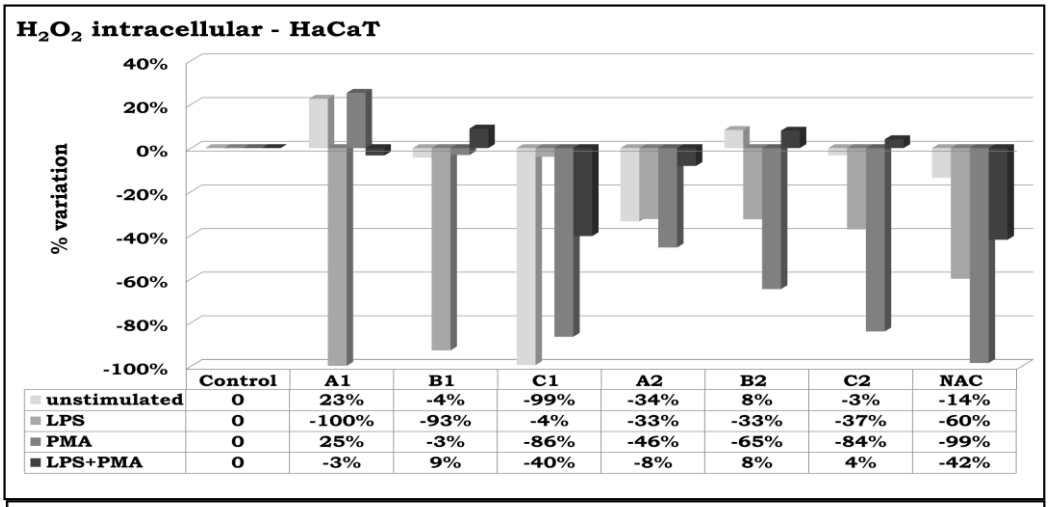
Sample	***SOD (U/ml)***				***Catalase - k(nmol/min/ml)***			
	unstimulated	***LPS***	***PMA***	***PMA+LPS***	***unstimulated***	***LPS***	***PMA***	***PMA+LPS***
N	40	57	83	77	3,27	6,68	3,38	7,18
A1(TES:Gb)	118	126	67	93	8,67	3,16	7,21	2,42
B1(TES:Gb)	162	184	85	33	2,41	8,04	2,01	1,49
C1(TES:Gb)	205	97	178	-6	6,86	8,59	1,18	2,37
A2(TES:Cs)	124	28	65	115	1,18	3,87	5,64	0,77
B2(TES:Cs)	123	74	58	117	6,60	5,51	0,35	10,76
C2(TES:Cs)	130	74	143	89	9,44	0,73	2,05	1,48
N-Ac-Cys	178	55	97	145	8,39	8,83	5,73	2,22

The combinations of active principles studied sustain the activity of oxidative enzymes by maintaining the cellular redox balance, thus counteracting the attack of free radicals, both by eliminating the superoxide anions under the action of SOD and the oxygenated water catalyzed by catalase. Also, combinations B1_TES:Gb (1: 1), B2_TES: Cs (1:1) and A2_TES:Cs (1: 9) activate SOD similar to the known antioxidant, N-Acetyl-Cysteine. In addition, B2_TES: Cs (1: 1) catalyzes the transformation of H_2O_2 decomposition, having multiple oxidative-reducing roles on the antioxidant enzyme system. In the case of the oxidative stimulation associated with the bacterial attack (LPS + PMA), the

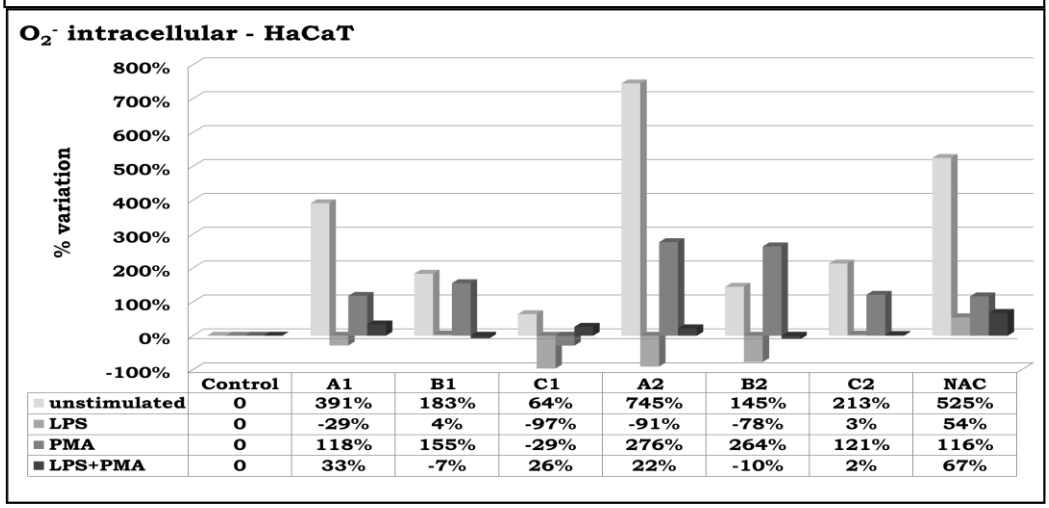
combinations A1, A2, B2 and C2 potentiate the activity of SOD, thus eliminating the superoxide anion from the system, without interfering in the process of decomposition of the oxygenated water.

1.7. Evaluation of intracellular oxidative stress by flow cytometry - simultaneous identification of intracellular oxygenated radicals (superoxide anion and hydrogen peroxide).

Evaluation of oxygenated radicals was performed on cell cultures grown under the same conditions as in the case of determination the SOD and Catalase enzymatic activity. The results are shown in the graphs below, as percent variation of the intracellular ROS expression in the presence of tested samples to the specific control – Fig.3,4.



	Control	A1	B1	C1	A2	B2	C2	NAC
unstimulated	0	23%	-4%	-99%	-34%	8%	-3%	-14%
LPS	0	-100%	-93%	-4%	-33%	-33%	-37%	-60%
PMA	0	25%	-3%	-86%	-46%	-65%	-84%	-99%
LPS+PMA	0	-3%	9%	-40%	-8%	8%	4%	-42%



	Control	A1	B1	C1	A2	B2	C2	NAC
unstimulated	0	391%	183%	64%	745%	145%	213%	525%
LPS	0	-29%	4%	-97%	-91%	-78%	3%	54%
PMA	0	118%	155%	-29%	276%	264%	121%	116%
LPS+PMA	0	33%	-7%	26%	22%	-10%	2%	67%

Fig. 3. Highlighting the effect of plant extracts studied in combination on oxygenated water and superoxide anion generated intracellularly - HaCaT cell line

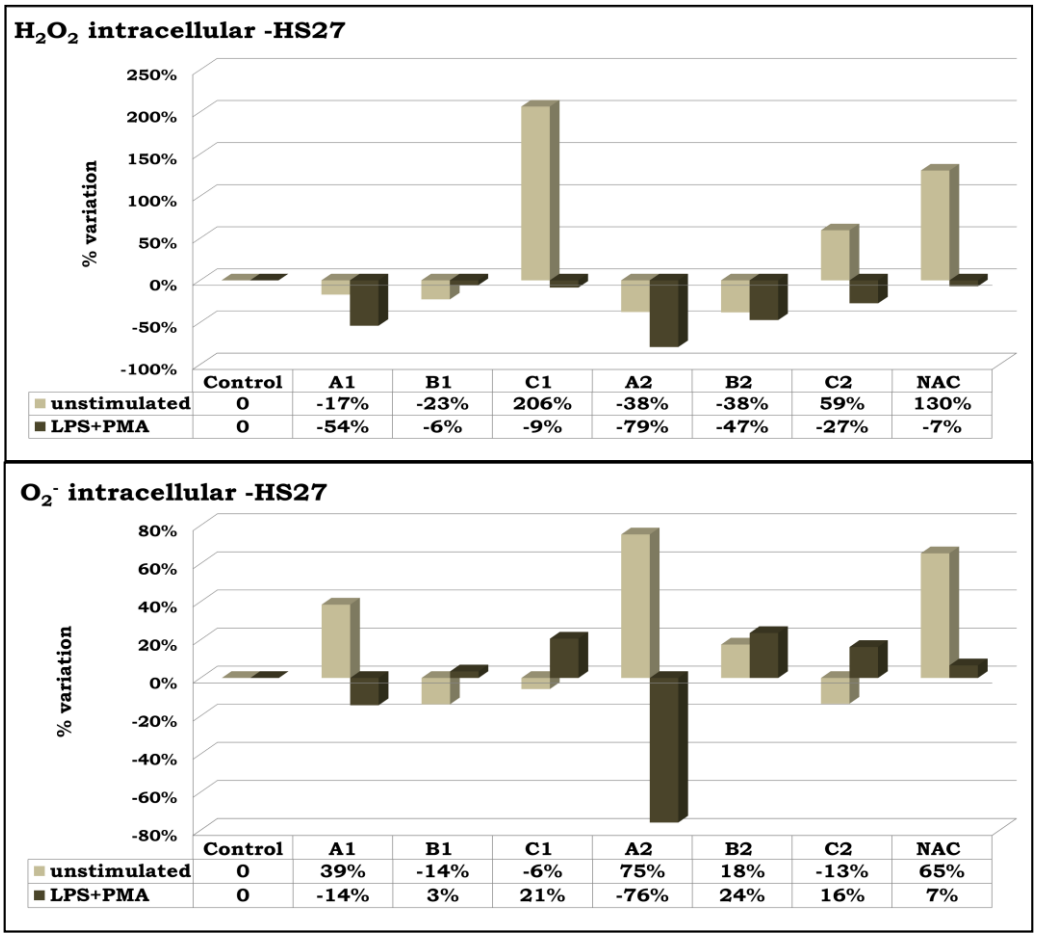


Fig. 4. Highlighting the effect of plant extracts studied in combination on oxygenated water and superoxide anion generated intracellularly – HS27 cell line:

The results indicate a signifiant antioxidant effect induced by the combinations of extracts tested on keratinocytes, both in the case of inflammation of bacterial origin (skin infections) and of the systemic inflammation associated with skin lesions, by the reducing effect on the oxygenated water and the superoxide anion. In the case of cells stimulated with LPS, only the A1, C1, A2 and B2 mixtures have a reducing effect on oxygenated water and intracellular superoxide, while upon stimulation with PMA a reduction of oxygenated water with superoxide anion accumulation is observed in the presence of A2, B2 and C2 mixtures. In dermal fibroblasts, in the case of simulation of endogenous oxidative stress (PMA), simultaneously with bacterial inflammation (LPS), the level of intracellular ROS is strongly low in the presence of combinations A2 and B2

Conclusions

This paper shows a multi-factorial approach of the oxidative stress phenomena, concerning the balance between antioxidant factors, free radicals and enzymatic systems, intrinsic /extrinsic mechanisms of modulation.

The antioxidant pathways investigated have outlined the synergistic potentiation of the antiradical and antioxidant effects for the extracts of marigold (Gb), chestnut (Cs) and grape marc (TES). These are explained by the composition in active principles (flavonoids, polyphenols), as well as by the relation structure / biological activity rigorously documented and demonstrated through studies at the cellular level.

The efficacy of the plant extracts in enhancing the antioxidant capacity of the skin cells has been demonstrated, suggesting their implication in skin pro - inflammatory and pro-oxidant phenomena prevention.

Acknowledgment

The study was performed as part of the AOSR project: “Capitalization in industry of residues from winemaking as food additives and antioxidants”.

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