Academy of Romanian Scientists *Volume 6, No. 2, 2017, pp. 5 - 14*
Annals Series on Biological Sciences ISSN 2285 – 4177

ORIGINAL PAPERS

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Valorification of Grape Marc by Obtain ing Bioactive Complexes Tested Through *In Vitro* Experimental Models

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Abstract

The most complete valorisation of plant raw materials as renewable natural resources is a current study direction involving a diversification of the identified and isolated active compounds from certain sources (parts of the plant, by-products, etc.), the efficiency of extractive technologies and the definition of a spectrum of relevant biological action. Starting from a widely spread raw material, wine-making waste (TES complex), the paper highlights the anti-inflammatory effect by monitoring the release into the growth medium of pro-inflammatory cytokines (IL6 and IL8). The study was performed on normal fibroblast and normal human keratinocyte cell lines, preceded by a cytotoxicity screening on the two cell lines. Induction of the inflammatory process in the in vitro experimental model was accomplished by simultaneous stimulation with TNFalpha and PMA and determination of extracellular release of pro-inflammatory cytokines. The results confirm an anti-inflammatory effect for the TES complex by inhibiting the major mediators of inflammation, suggesting multiple pharmaceutical and cosmetic applications of these compounds.

Keywords*:* grape marc, cytotoxicity, cytokines, inflamation

Introduction

Grape marc is the residue from the winemaking process (shells, seeds, tails) and is an important source of polyphenolic compounds (hydroxycinnamic acid and hydroxybenzoic acid derivatives, flavonoids, anthocyanins, tannins, proantocianidines, stilbens) [1]. Among these, flavonoids are the most abundant and widely studied, with biological properties, including antioxidant, anti-inflammatory, anticancer, antimicrobial, antiviral, cardioprotective, neuroprotective and hepatoprotective activities [2].

Polyphenols decrease chronic inflammation either by modulating inflammatory pathways or by reducing levels of reactive oxygen species. It has also been shown that grape seed proanthocyanidins have high anti-inflammatory action because they have the role of capturing free radicals, preventing lipid peroxidation, and inhibiting the formation of proinflammatory cytokines [3]. The use of marc as an important source of bioactive phytocompounds with applications in the pharmaceutical, cosmetic and food sector is an efficient, profitable and environmentally friendly alternative to the residues generated during the winemaking process.

The aim of this study was to evaluate cytotoxic profile and to determine anti-inflammatory activity by highlighting the effect of TES extract on proinflammatory cytokines (IL-6, IL-8) on normal human keratinocyte (HaCaT) and dermal fibroblast (HS27) human cell lines.

Materials and Methods

Materials

Vegetable material: vegetable residues resulting from the winemaking process. After a 7-day fermentation period, the grape marc was dehydrated by maintaining at 45°C and continuously vacuum, then ground to obtain the TES extract.

Standardized cell lines: Fibroblast (normal cell line HS27) - cells with high proliferative capacity, cell cultures reach confluency relatively quickly (80% confluence in 3 days). Cells were cultured in monolayer in DMEM (Dulbecco's

Modified Eagle's Medium / Nutrient Mixture F-12 Ham, code: D8437, Sigma-Aldrich) supplemented with 10% fetal bovine serum (code: F7524, Sigma-Aldrich) used in tests between passages 20-40. Keratinocit (HaCaT) - epithelial squamous cell with a remarkable regenerative potential, which undergoes a differentiation process during its migration from the level of the germinative layer to the cortical layer, descuamative. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (code: 30-2002, ATCC®) supplemented with 10% fetal bovine serum and used in experiments at passages 40-50.

Chemical Substances: The control agents used, were N-acetyl-cysteine (code: SC-202232, Santa Cruz Biotechnology) as antioxidant and dexamethasone (code: D4902, Sigma-Aldrich) as anti-inflammatory agent. For cytotoxic potential evaluation were used: CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) kits - for the determination of metabolic activity (MTS) and CytoTox 96® Non-Radioactive Cytotoxicity Assay from Promega - for the release of lactate dehydrogenase (LDH) in extracellular medium. For the determination of cytokines for extracellular cytokines by flow cytometry, the BD Cytometric Bead Array (CBA) - Human Inflammatory Cytokines kit (BD Pharmingen) kit was used.

Methods

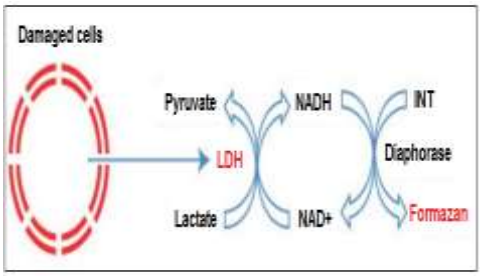
a) Evaluation of the cytotoxic effect on a cell culture.

The cytotoxic effect of a substance on a cell line is evaluated by establishing the correlation between decreased cell viability (MTS test) and enhancing enzyme activity in the culture medium (LDH assay). To assess the cytotoxicity of the samples studied, the cells were exposed to increasing concentrations of the test products over a period of time, depending on the cellular metabolic characteristics.

Reduction of MTS, cell viability marker: Treatment of MTS cells, tetrazolium 3 (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2H-tetrazolium tetrazolium salt allows evaluation of oxidative metabolism and response of a cell population to external factors that may have a positive or negative effect on cell life in culture. For this reason, the MTS test is used in cell

viability and proliferation studies [4]. Conversion of MTS to water-soluble formazan occurs under the action of enzymes (dehydrogenases) found in metabolically active cells. The amount of formazan produced, measured as absorbance at 490 nm, is directly proportional to the number of live cells in the culture [5,6].

Release of lactate-dehydrogenase in cell medium, cytotoxicity marker: Lactate dehydrogenase (LDH) is a cytosolic enzyme present in all cells which, under normal physiological conditions of the plasma membrane, remains in the cytoplasm. The in vitro release of LDH provides a precise way of measuring cell membrane integrity and, implicitly, cell viability [7]. The release of LDH in the cell culture supernatant is measured by a test in which two coupled enzymatic reactions catalyzed by LDH and diaphorase are carried out which result in the conversion of a tetrazolium salt into a red formazan compound [8].

a. 

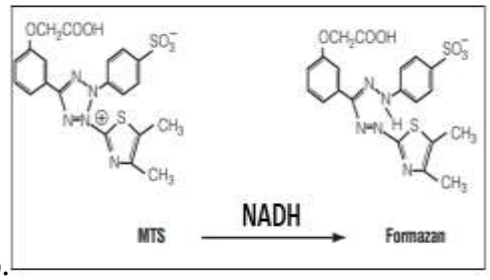
b. 

Figure 1. Cytotoxicity assay: a. LDH eliberation [9]; **b.** MTS tetrazoliun reaction [10]

b) Determination of extracellular pro-inflammatory cytokines by flow cytometry and use of capture beads:

The assay uses a series of discrete fluorescence intensity particles for the simultaneous detection of several soluble analytes (inflammatory cytokines). Each particle (beads) of the kit has a capture surface coated with antibodies specific for IL-8, IL-6 [11]. The capture beads, the conjugated detection antibodies and the recombinant standards or assay samples are incubated together to form a sandwich complex that is visualized in APC-A / PE-A coordinates following acquisition of flow cytometry. Analysis of fluorescence histograms and

interpolation of values on calibration curves is performed with FCAP Beads Array software (fig.2).

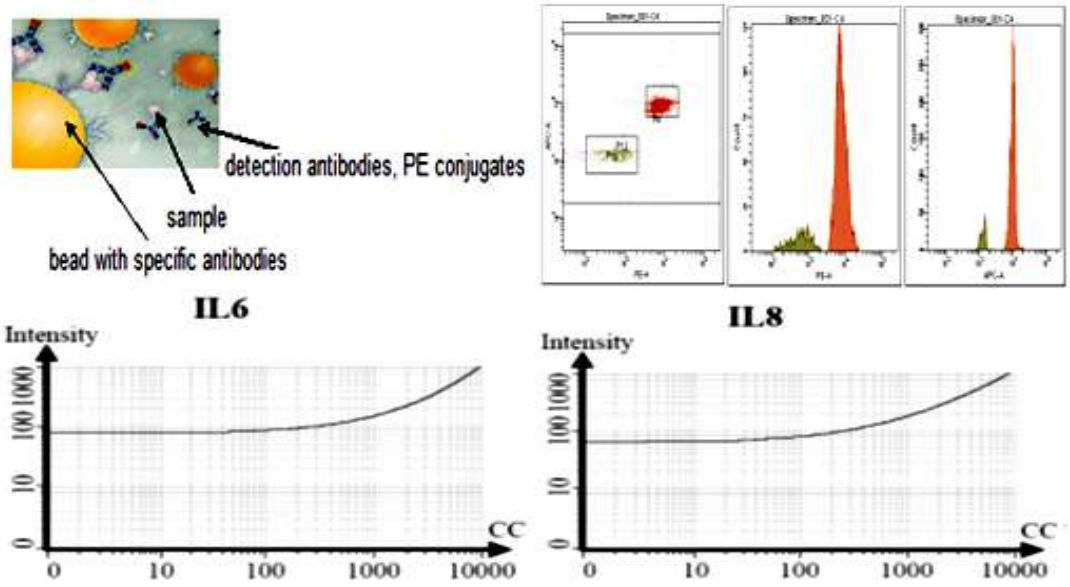
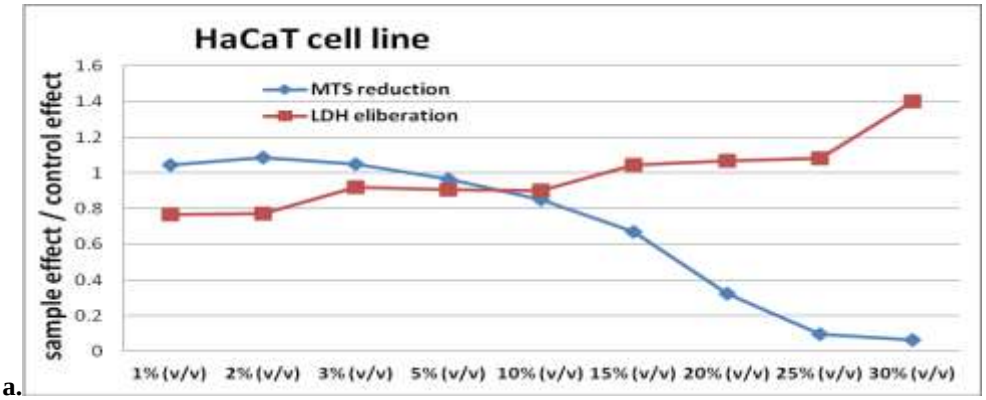


Figure 2. IL8 and IL6 flow cytometry detection [11]

Results and Discussions

Evaluation of the cytotoxic effect of TES extract by establishing the correlation between cell viability decrease (MTS assay) and enhancing enzyme activity in the culture medium (LDH assay) using ELISA technique.

a.



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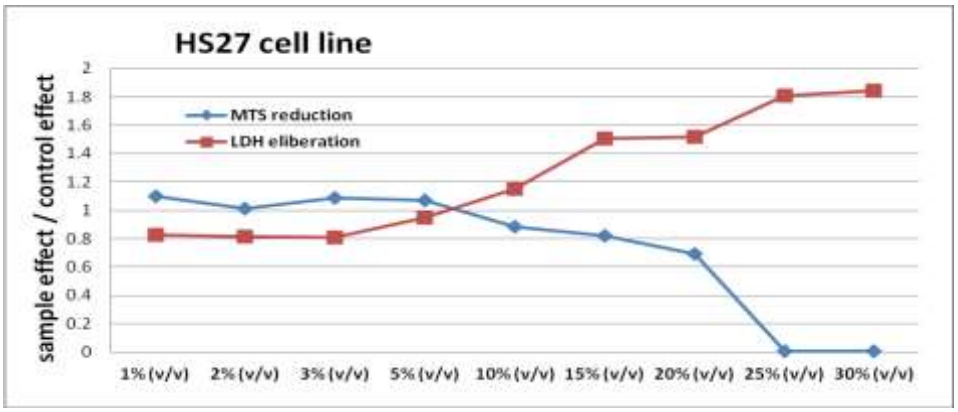


Figure 3. Evaluation of the cytotoxic potential of TES extract revealed by MTS and LDH assays on HaCaT - keratinocyte (a.) and HS27 - fibroblast (b.) cell lines. The cells were allowed to adhere for 24h (7000 cells / well) and treated for 48 hours with the test substance according to the protocol for the specific reagent kit (MTS / LDH). All concentrations of active principle were tested in triplicate.

On the HaCaT (a.) cell line, the cytotoxicity threshold is 7.5%, this concentration increases the amount of lactate dehydrogenase released in the medium, while the reduction in MTS decreases, indicating a loss of cell membrane integrity and decreased cell viability. On the HS27 (b.) Cell line, the TES complex has a cytotoxic effect starting at a 5% concentration. Concentrations below this limit has superior ratio of sample/ control effects, the metabolic activity evaluated by MTS technique being higher, suggesting an activation of cellular metabolism induced by extract components.

Cytometric evaluation of extracellular release of pro-inflammatory cytokines (IL-6 and IL-8) by keratinocytes and fibroblasts simultaneously stimulated with TNF-α and PMA.

Inflammation is the protective response of tissues against cellular lesions, irritation and pathogenic invasions. Chronic inflammation is considered to be the primary mediator in the development of chronic diseases such as cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, arthritis, autoimmune and pulmonary diseases. Intensive production and secretion of cytokines and proinflammatory chemokines, once started, may form concentration gradients in the affected tissues, which may lead to the amplification of the initial

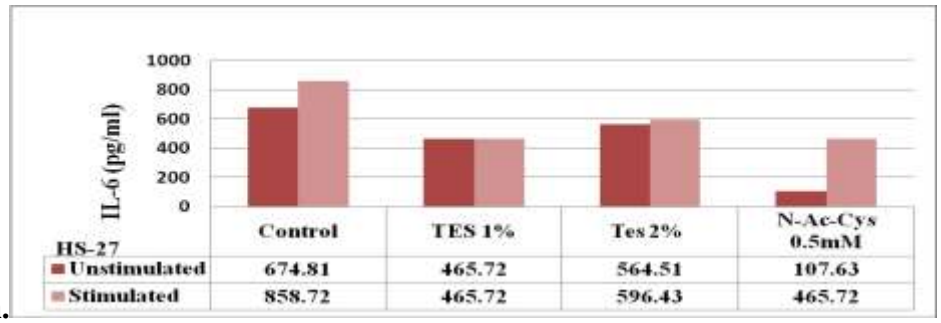
inflammatory response. IL-6 is a cytokine involved not only in inflammation and response to infections, but also regulating metabolic and regenerative processes [12]. Tumor promoters, proinflammatory cytokines, endotoxins and synthetic protein inhibitors can modulate cell cycle kinetics of different cell types, stimulate the production of reactive oxygen species and induce keratinocytes to produce interleukin-8 (IL-8), a strong chemotactic for polymorphonuclear neutrophils Lymphocytes T [13]. IL-8 is involved in neutrophil activation and is released from several types of cells in response to inflammation, including monocytes, macrophages, neutrophils and intestinal, kidney, placenta and bone marrow cells [14].

To evaluate the anti-inflammatory effect of the extracts, the following experimental systems were performed on human fibroblasts and human keratinocytes cultures from standardized lines: cytokine IL6, IL8 detection in the culture supernatant; pro-inflammatory stimulatory conditions differentiated with PMA 0.1 μM and TNF-α 15 ng/ml; positive control N-acetyl-cysteine (antioxidant) and dexamethasone (anti-inflammatory).

In the experimental model used, the effect of TES extract was compared with NAC on cell line HS27 and with NAC and Dexamethasone on the HaCaT cell line. Tumor necrosis factor (TNF-α) is a signaling protein (cytokine) involved in systemic inflammation and is one of the cytokines responsible for the acute phase reaction. TNF-α is a central regulator of inflammation, and its antagonists can be effective in treating inflammatory conditions, where TNF-α plays an important pathogenic role [15]. PMA (Phobol 12-myristate 13-acetate) also known as 12-O-tetradecanoylphorbol 13-acetate (TPA) is a specific activator of Protein Kinase C (PKC) and NF-kB. PMA affects to a large extent the cells and tissues, being known as a pro-oxidative activity stimulator [16]. N-acetylcysteine (NAC) is an L-cysteine precursor used to increase glutathione production. Through the glutathione buffering activity, NAC has antioxidant action.

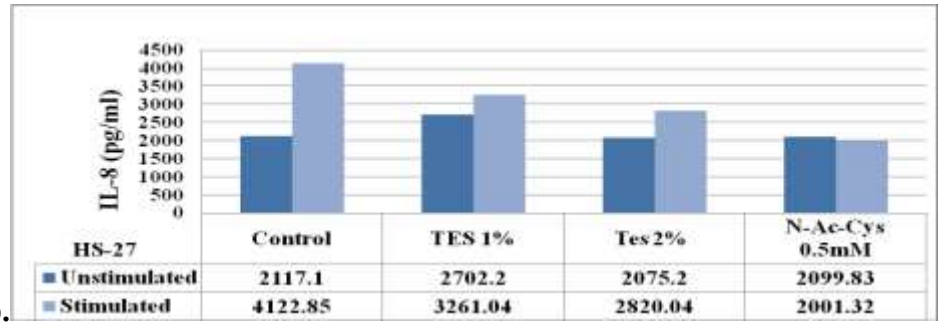
The experimental results are presented in fig.4.

a.



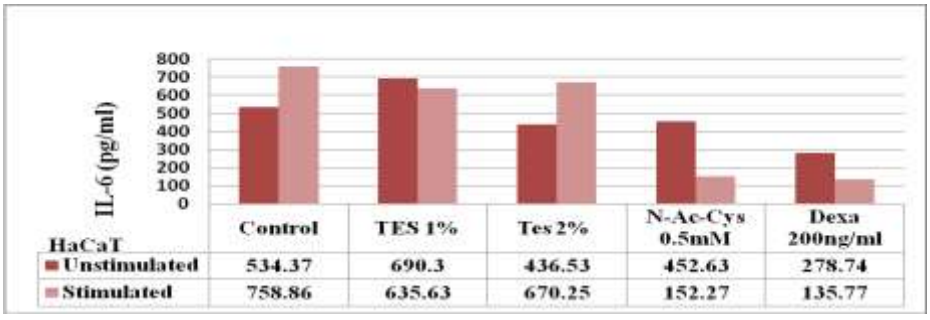
HS-27	Control	TES 1%	Tes 2%	N-Ac-Cys 0.5mM
Unstimulated	674.81	465.72	564.51	107.63
Stimulated	858.72	465.72	596.43	465.72

b.



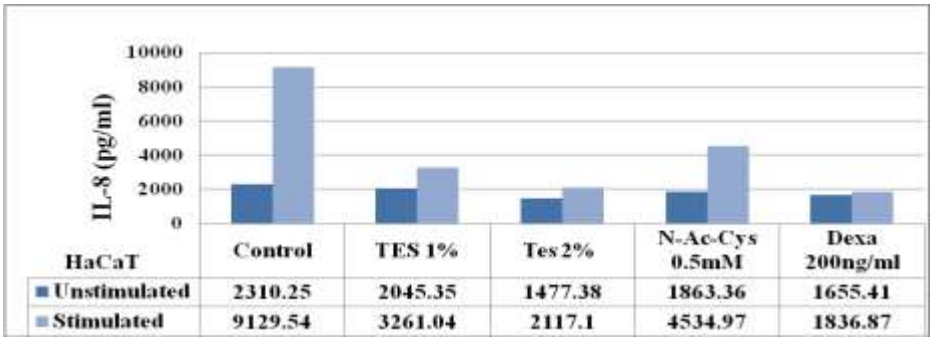
HS-27	Control	TES 1%	Tes 2%	N-Ac-Cys 0.5mM
Unstimulated	2117.1	2702.2	2075.2	2099.83
Stimulated	4122.85	3261.04	2820.04	2001.32

c.



HaCaT	Control	TES 1%	Tes 2%	N-Ac-Cys 0.5mM	Dexa 200ng/ml
Unstimulated	534.37	690.3	436.53	452.63	278.74
Stimulated	758.86	635.63	670.25	152.27	135.77

d.



HaCaT	Control	TES 1%	Tes 2%	N-Ac-Cys 0.5mM	Dexa 200ng/ml
Unstimulated	2310.25	2045.35	1477.38	1863.36	1655.41
Stimulated	9129.54	3261.04	2117.1	4534.97	1836.87

Figure 4. Proinflammatory cytokines (IL-6, IL-8) eliberation on HS-27 (a, b) and HaCaT (c, d) cell lines in the presence of TES extract

In TES-treated cells, inhibition of IL-6 cytokine is observed under both normal and inflammatory conditions (69% in stimulated fibroblasts and 88% in stimulated keratinocytes).

Under non-specific inflammatory conditions simulated with PMA and TNFα, inhibition of IL-8 cytokine was observed in cells treated with 2% TES as compared to the cell control (68% in fibroblasts and 23% in keratinocytes).

Conclusions

The grape marc proves to be a reliable source of bioactive substances, with a convenient profile of toxicity on HaCaT and HS27 standardized cell lines. The 7% (v/v) concentration for keratinocytes and 5% (v/v) for fibroblasts are reasonable limits that recommend its safety use in topical products. As well as, the 1 – 2% (v/v) is the active dose inhibiting the main inflammation promoters' release, IL6 and IL8, on both keratinocytes and fibroblasts. All these data sustain the capitalization of vegetal waste, especially the grapes one, as resources for raw materials in pharmaceutical and cosmetic industry.

Acknowledgment

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

References

[1] Agustin-Salazar s., Medina-Juárez L.A., Soto-Valdez H., 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, 2014, 20, 208–213.

[2] Korkina L., Kostyuk V., de Luca C., Pastore S., Plant phenylpropanoids as emerging anti-inflammatory agents, Mini Rev. Med. Chem., 2011, 11, 823–835.

[3] Georgiev V., Ananga A., Tsolova V., Recent Advances and Uses of Grape Flavonoids as Nutraceuticals, Nutrients, 2014, 6, 391-415

[4] 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, 1997, 124(3), 179-92.

[5] Barltrop J.A. et al. 5-(3-carboxymethoxyphenyl)-2-(4,5-dimenthylthiazoly)-3-(4-sulfophenyl) tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators, Bioorg. Med. Chem. Lett., 1991, 1, 611–4.

[6] Riss T.L., Moravec R.A., Comparison of MTT, XTT, and a novel tetrazolium compound for MTS for in vitro proliferation and chemosensitivity assays. Mol. Biol. Cell (Suppl.), 1992, 3, 184a.

[7] Fotakis G., Timbrell J.A., In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride, Toxicology Letters, 2006, 160, 171-177.

[8] Allen M., Millett P., Dawes E., Rushton N., Lactate dehydrogenase activity as a rapid and sensitive test for the quantification of cell numbers in vitro, Clin Mater., 1994, 16(4):189-94.

[9] Decker T., Lohmann-Matthes M.L., A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity, J Immunol Meth, 1988, 115:61-9.

[10] Cory A.H., Owen T.C., Barltrop J.A., Cory J.G., Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture, Cancer Commun., 1991, 3, 207-12.

[11] Cook E.B., Stahl J.L., Lowe L., Chen R., Morgan E., Wilson J., Varro R., Chan A., Graziano F.M., Barney N.P., Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics, Journal of Immunological Methods, 2001, 254, (1–2), 109-118.

[12] Scheller J., Chalaris A., Schmidt-Arras D., Rose-John S., The pro- and anti-inflammatory properties of the cytokine interleukin-6, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2011, 1813, 5, 878-888.

[13] Wilmer J.L., Luster M.I., Chemical induction of interleukin-8, a proinflammatory chemokine, in human epidermal keratinocyte cultures and its relation to cytogenetic toxicity, Cell Biology and Toxicology, 1995; 11, 37-50.

[14] Bickel M., The role of interleukin-8 in inflammation and mechanisms of regulation, J Periodontol., 1993, 64, 456-60

[15] Esposito E., Cuzzocrea S., TNF-alpha as a therapeutic target in inflammatory diseases, ischemia-reperfusion injury and trauma, Curr Med Chem., 2009,16(24):3152-67.

[16] DeChatelet L.R., Shirley P.S., Johnston R.B. Jr., Effect of phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leukocytes, Blood. 1976, 47(4):545-54.