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Vascular anti-inflammatory effects of natural compounds from *Aesculus hippocastanum and Hedera helix*

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Abstract:

In inflammatory processes and wound healing, the fundamental mechanisms of progression are the dynamic change of cellular adhesion and leucocytes migration (expression of adhesion molecules (ICAM and VCAM)), the release of pro-inflammatory cytokines IL-6 and IL-8 in vascular endothelial cells and edema occurrence due to increased vascular permeability- VEGF (vascular endothelial growth factor) signaling pathway.

The aim of our studies was to determine the anti-inflammatory effect at vascular level of some compounds isolated from Hedera helix and Aesculum hypocastanum, through flow cytometry studies of VCAM-1 and ICAM-1 expression, determination of the pro-inflammatory cytokines IL6 and IL8 in extracellular environment and the VEGF factor. The inflammation was induced by two different stimuli: LPS, lipopolysaccharide that mimics the bacterial infection, respectively TNF-α nonspecific stimulus for the acute phase systemic inflammation.

Both compounds inhibit IL6 and IL8 secretion and block the VEGF pro-angiogenic factor, but have no significant action on the expression of adhesion molecules in bacterial inflammation.

During the stimulation with TNF-α, the Hedera helix extract has a concerted effect inhibiting in a dose-effect manner the IL6 and IL8 cytokines, ICAM and VCAM expression and VEGF. Aesculum hypocastanum extract enables the inhibition of IL6 and IL8 and the ICAM and VCAM expression.

Key words: vascular endothelium, ICAM, VCAM, IL6, IL8, VEGF, inflammation

Introduction

In inflammatory processes and wound healing, one of the fundamental cellular properties is the dynamic change of cellular adhesion and leucocyte migration. The inflammatory stimuli influence the expression of adhesion molecules (ICAM and VCAM) and release of pro-inflammatory cytokines IL-6 and IL-8 in vascular endothelial cells. Intercellular adhesion molecule-1 (ICAM-1) and vascular molecule-1 (VCAM-1) mediate adhesion and transendothelial migration of leucocytes at inflammation locus. The released cytokines network maintains propagation of proinflammatory pathways, being an important target for anti-inflammatory products.

One of the fundamental mechanisms of inflammation is impaired progression of leukocyte extravasations across vascular endothelium and infiltration into adjacent tissues (Peschen M.T. et all, 1999)[1]. Infiltrated leukocytes secrete high levels of mediators of inflammation, perpetuating the inflammatory response, which results in degradation of inflamed tissue. For tissue invasion by inflammatory cells is decisive their transmigration along micro vascular endothelium, a process mediated by adhesion molecules from CAM class (Cellular Adhesion Molecules): ICAM and VCAM expressed on endothelial cells and their corresponding receptors from leukocytes. There are dermatological diseases such as atopic dermatitis, psoriasis, scleroderma and multiform erythema in which the expression of

these molecules is significantly increased in association with skin damage and poor wound healing.

Although expression of both molecules is increased in inflammation, VCAM-1 has a predominant role in initiating this process. ICAM-1 is expressed mainly in micro vessels, lacking in large veins or arteries. During the vascular inflammatory process, endothelial cells play an important role, producing cytokines and adhesion molecules involved in its evolution. Endothelial activation is a critical event in the inflammatory process, being associated with chemotactic cytokines (chemokines) that cause leukocytes diapedesis from the blood circulatory system to the site of inflammation (Goebeler M.T. et all,1997) [2]. IL6 plays a key role in the acute phase response, dictating the transition from acute chronic phase by changing the leukocyte infiltrate and stimulate T and B lymphocytes to promote chronic inflammation. IL8 is involved in acute inflammation, in the recruitment and activation of neutrophils (migration to the site of inflammation, release of granular enzyme that degrades the connective tissue), functions as an autocrine growth factor involved in inflammation, infection, cellular stress, tumor developing, but also as angiogenetic factor in endothelial cells. Thus, agents with inhibitory activity on the synthesis and release of IL-6 and IL8 may confer protection by stopping the inflammatory pathways mediated by these interleukins.

Besides the cellular expression of endothelium - monocytes adhesion factors, another process triggered by inflammatory stimuli is the extracellular secretion of cytokines with a characteristic role in the progression of inflammation. Therapeutic intervention on both inflammatory pathways leads to sustained effects of stopping the inflammation at vascular endothelium level.

Another aspect of tissue inflammation is the appearance of edema due to increased vascular permeability.

Also, although extensively studied correlated with tumor invasion, angiogenesis is a marker of chronic inflammatory diseases, and is a relationship between the main promoters of VEGF (vascular endothelial growth factor) family factors and disease progression (Celletti F.L.et all,2001, Paleolog E.M.,2002, Kanazawa S.T.,2001)[3,4,5].

The VEGF is a multifunctional cytokine required for the normal development of the vascular system (Kuldo J.M., et all , 2005) [6]. The VEGF mechanism of action is channeled in the following ways: vasodilatation (the blood flow increasing), the angiogenesis stimulation and the vascular permeability increasing, the main effect being the nutrients intake increasing and the toxic compounds removing from a degraded tissue (Bates D.O.,et all, 2002) [7]. The "in vitro" studies showed the VEGF action as a homo-dimer that binds to three different receptors, thus stimulating the endothelial cell mitosis, migration and increased endothelial monolayer permeability, stimulating even the microtubules formation (Iwasaka C.,et. All, 1996) [8]. Based on the research at the molecular level of the VEGF action anti-cancer, rheumatoid arthritis, or diabetic retinopathy drugs have been developed, having this factor inhibition therapeutic target and thus decreasing the vascular permeability and edema.

In addition to traditional anti-inflammatory synthesis drugs (eg, dexamethasone) with known role in reducing the expression of ICAM-1 and pro-inflammatory cytokines secreted by endothelial cells (Wen Chieh C., et all, 2002) [9], recent research highlights the protective role of phytocompounds (Guohua G.,et all, 2010) [10]. For example, β escine acts in angiogenesis and endothelial functionality (Lindahl T.L.,et all, 2003, Wang X.H., et all 2008)[11,12], while saponins occur in vascular protection and decrease permeability. As well as, our previous studies have shown the strong impact of natural active principles therapy on inflammatory status of normal cells (Dumitriu et all., 2012)[13] and most important, in cancer therapy (Olariu et all, 2011) [14].

The purpose of conducted experiments was to reveal the vascular anti-inflammatory effects of compounds isolated from *Hedera helix* and *Aesculum hypocastanum* through functional, endothelial cells analysis of the following parameters:

- assesment of monocyte – endothelium adhesion inhibition through flow cytometry studies of VCAM-1 and ICAM-1 proteic expression;
- assessment of extracellular release of IL6 and IL8 pro-inflammatory cytokines by stimulated endothelial cells
- theVEGF pro-angiogenic factor determination as an indicator of the vascular permeability.

There have been designed five succesive experiments leading to the establishment of relevant conditions of stimulation and vascular effects of Dermo-Es and Dermo- HdC isolated from *Aesculum hypocastanum* and *Hedera helix.* Evaluations were done compared with a positive control: dexamethasone 0.6μg/ml, a potent, synthetic anti-inflammatory agent (Hettmannsperger U.,et all 1992) [13].

Materials and Methods

Bioactive compounds:

Dermo-Es - from ***Aesculum hypocastanum***

Laboratory procedure for obtaining "Dermo Es" bioproduct involves selective extraction and separation of the compound from vegetal raw material (seeds of *Aesculum hypocastanum)*, followed by a purification step in order to obtain a preparation as a white powder containing min 70%. In order to purify Dermo-Es, the dry extract was dissolved in a mixture of n-propanol: ethyl acetate: water, cold saturated with borax, followed by separation of interest fraction on aluminum oxide column. Chromatographic analysis of the compound is shown in the figure below:

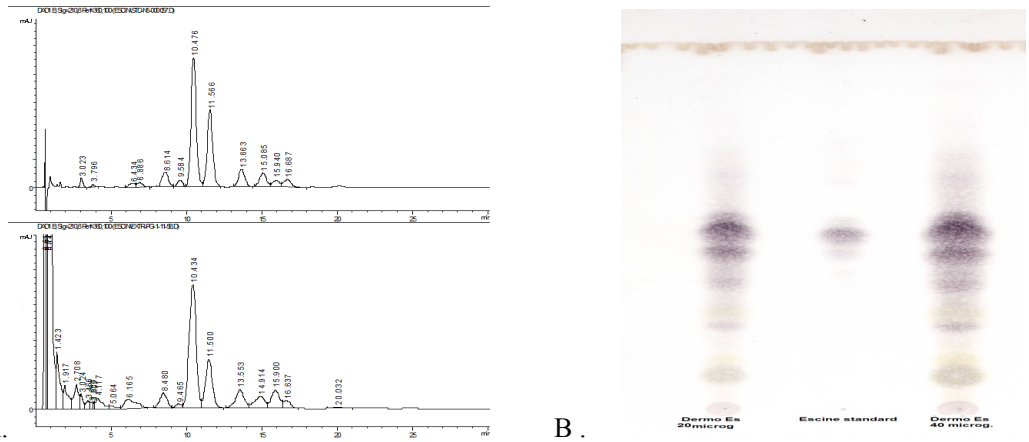

Figure 1. A: HPLC identification of Dermo-Es: chromatographic prophile of standard solution (up) and chromatographic prophile of sample solution (down).

B. Thin layer chromatogtaphy of Dermo –Es:the spot in the middle represent the standard solution

Dermo-HdC – from *Hedera helix*

Laboratory procedure for obtaining "Dermo HdC" bioproduct involves selective extraction and separation of bidesmosidic saponines from vegetal raw material (leafs of

Hedera helix), followed by a purification step in order to obtain a preparation as a yellow-white powder containing 60-66% triterpenis bidesmosidic saponine. Chromatographic analysis of the compound is shown in the figure below:

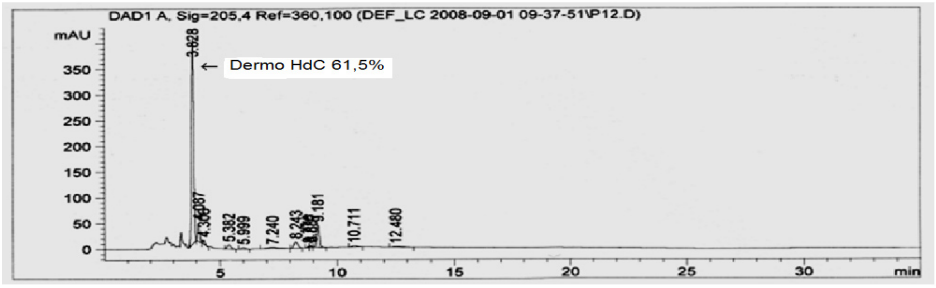

Figure 2: HPLC identification of Dermo -HdC:**.**

Cell culture: HUVEC cell line (Human umbilical vascular endothelial cell, ATCC PCS-100-). All the experiments used 2-6 passages. The cells were harvested after 24h of adhesion and 48h, treatment with test substances and positive control (Dexamethasone 0.6µg/ml). The culture medium was RPMI with 10% bovine fetal serum and 1% antibiotic / antimicotic solution. The cells were maintained in 5%CO_2 athmosphere, at 37^oC. The passages were done at confluence, through enzymatic detach with Tripsine / EDTA.

Stimulation conditions:

The experimental systems consisted in differentiated stimulation with LPS, lipopolisacharide wich mimics bacterium infection, respectively TNF-α- unspecific stimulus for systemic inflammation. LPS is a major component of gram negative bacteria, acting as endotoxine at animal organism level, inducing a strong immune response and producing pro-inflammatory cytokines secretion (Yoshihiko S et all, 2008) [14]. TNF-α is a part of cytokines group that stimulate acute phase inflammation reaction. Specific doses and time of stimulation were established through preliminary experiments presented in Result and Discussion section.

Methods for cellular parameters analysis:

- **Evaluation of protein expression of VCAM and ICAM through flow cytometry-**

Staining method:

Cells are harvested through tripsinisation (Tripsin/EDTA 0.1g% - Sigma) and are staining with specific fluorescent antibodies for ICAM-1 and VCAM-1, as follow:

- APC Mouse Anti-Human CD54 for ICAM-1 (intercellular-adhesion molecule).
- PE Mouse Anti-Human CD106 for VCAM-1 (vascular-cell-adhesion molecule).

10^6 cells are staining with 20µl antibody (BD Pharmingen), followed by incubation at room temperature, in the dark, 20 minutes. Cells are washed with 2ml staining Buffer (BD Pharmingen). The pellet is resuspended in 0.5mL Staining Buffer and the samples are ready to be acquired at the flow cytometer.

The flow cytometer FACS CANTO II and FACS Diva 6 software are used to acquire and analyze "dot plot" representation for cell population visualization and fluorescence histograms for ICAM-1 (APC parameter), respectively VCAM-1 (PE - parameter). The results are evaluated comparing the mean of fluorescence channel APC or PE respectively.

- **Extracellular cytokines analysis – Flow cytometry detection of soluble proteins** through BD™ Cytometric Bead Array (CBA),a flow cytometry application that allows users to quantify multiple proteins simultaneously. Each capture bead in the kit has been conjugated with a specific antibody. The detection reagent provided in the kit is a mixture of

phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte (in our particular case IL6 and IL8).

When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, sandwich complexes (capture bead+ analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector (Cook E.B.,et all, 2001) [15]. The analysis of the results (standard curve for each cytokine and concentration calculation) is done with FCAP Beads Array software.

Expression of the pro-angiogenetic factor VEGF: The cell supernatant is harvested and processed to quantify VEGF by using the fluorescent labeled beads technique analyzed by flow cytometry.

Materials and method: Human VEGF Flex Set (BD Bioscience);Human Soluble Protein Master Buffer Kit (BD Bioscience). The principle of the method is the same that those used for IL6 and IL8 detection, previously described: the capture beads with certain fluorescence intensity are related to specific antibodies for a specific soluble protein, in this case VEGF. The data analysis by FCAP Array software provides a calibration curve which allows the concentrations of this glycoprotein to be determined based on the fluorescence intensity estimated by flow cytometry. The calibration curve corresponding to these series of experimental determinations is shown in Figure 2, with the analyte concentrations represented on the abscissa between 5 and 5000 pg/ml and the intensity of fluorescence on the ordinate.

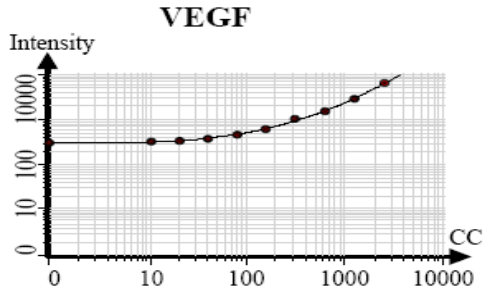

Figure 2. Calibration curve for VEGF (concentration versus fluorescence intensity)

Statistics: The experimental data represents the arithmetic average from 5 experiments. The graphics represent the variation percent from the cellular control, based on the following formula:

$$\% \text{ of variation} = [(\text{Value}_{\text{sample}} - \text{Value}_{\text{culture/solvent control}}) / \text{Value}_{\text{culture/solvent control}}] * 100$$

Results and Discussions

Endothelial cell culture is checked before the experiments for the expression of von Willebrand factor, a specific glycoprotein playing a key role in endothelium – thrombocytes adhesion, in haemostatic and wound healing process (Wissink M.J.B et. all, 2001) [16]. The expression of this protein assures the functionality of endothelial cells, being a well – known marker for the specificity of this type of cells (Bevilacqua M.P et all, 1987) [17]. The cellular staining is done with fluorescent antibodies and the samples are subsequently analyzed by flow cytometry (Kishimoto T.et all, 1997) [18]. Cells expressing Von Willebrand Factor emit the fluorescence response in FITC coordinate and can be evaluated through mean of fluorescence channel of the histogram (Figure 3).

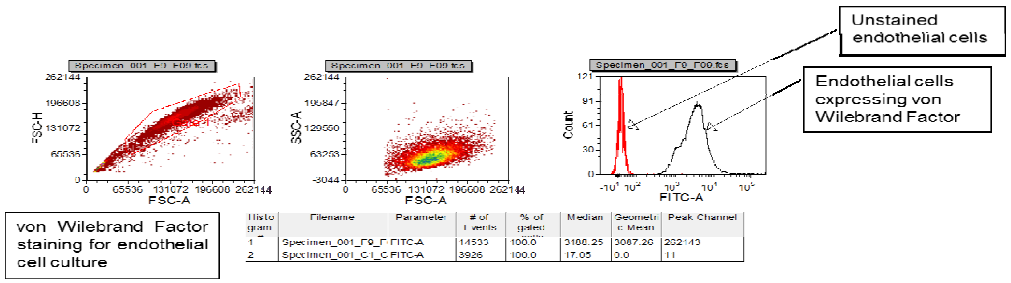

Figure 3. Flow cytometry checking of HUVEC cell line specificity (expression of von Wilebrandt Factor)

We shall follow the relevant parameters for inflammatory status:

- LPS (bacterial origin) stimulation, followed by ICAM expression analyze (characteristic for microvasculature), and IL6 and IL8 inflammatory cytokines,
- TNF-α, stimulation - systemic, unspecific stimulus, followed by VCAM and ICAM expression analysis, as well as IL6 and IL8 inflammatory cytokines.

We tested many TNF-alpha doses between 100ng/ml and 2ng/ml and LPS between 0.1μg/ml and 10μg/ml in order to establish the incubation time and the optimal doses for HUVEC cell line stimulation.

The results are presented in the table 1.

Table 1. Relevant parameters for vascular inflammatory status and doses of stimuli generating the inflammation

	TNF α 2ng/ml 5h of action	**TNF α 33ng/ml 5h of action**	**TNF α 100ng/ml 5h of action**	**LPS 1μg/ml 16h of action**	**LPS 5 μg/ml 16h of action**	**LPS 10 μg/ml 16h of action**
	%variation compared with untreated control	%variation compared with untreated control	%variation compared with untreated control	%variation compared with untreated control	%variation compared with untreated control	%variation compared with untreated control
ICAM	44.2	8.3	Death cells (Tripan Blue staining)	28.4	-8.9	-5.5
VCAM	19.6	4.2	Death cells (Tripan Blue staining)	-5.8	-7.7	47.4
IL6	315.8	11.3	9.8	297.4	15.7	40.4
IL8	283.4	14.2	-16.4	233.1	18.5	37.4

The better stimulation is obtained for TNF-α 2ng/ml, for all inflammatory parameters. LPS acts as an optimum stimulus for bacterial infection in acne lesions (ICAM- for micro vessels, IL6 and IL8 – significant parameters) at 1μg/ml.

The following data will present the results on the main anti-inflammatory effects function of the type of stimulation:

I. 1. Anti-inflammatory effects – LPS stimulation (mimics the bacterial infection in acne lesions, venous ulcerations, wounds super-infections.)

The values for the main interest parameters and the percent of variation are presented in tables 2-7. The ICAM expression is evaluated as median of fluorescent channel of corresponding flow-cytometry histograms, IL6, IL8 cytokines and VEGF factor are expressed in pg/ml (concentration in extracellular medium).

Table 2.ICAM adhesion molecules expression: **Dermo-Es** – comparing with cellular control and positive control

Tested substance	**Unstimulated cells**		**LPS 1µg/ml stimulated cells**	
	ICAM (median of fluorescent channel)	% of variation	ICAM (median of fluorescent channel)	% of variation compared with the unstimulated sample
Cellular control	10882,6		13974,4	28,4
Dermo Es 2µM	12494,9	14,8	13453,7	-3,7
Dermo Es 1µM	12784,2	17,5	12761,5	-8,7
Dermo Es 0,5µM	13008,5	19,5	12787,8	-8,5
Dexamethasone 0,6ug/ml	10235,8	-5,9	11097,4	-20,6

Dermo-Es didn't signifficantly change the ICAM expression. (% of variation below 10%).

Table 3 : Dermo-Es effects on extracellular release of pro-inflammatory cytokines

Tested substance	**Unstimulated cells**				**LPS 1µg/ml stimulated cells**			
	IL6 (pg/ml)	% of variation	IL8 (pg/ml)	% of variation	IL6 (pg/ml)	% of variation	IL8 (pg/ml)	% of variation
Cellular control	2054.5		1446.6		8166.1	297.5	4819.5	233.2
Dermo Es 2µM	7896.8	284.4	4965.5	243.2	6922.8	-15.2	4483.1	-7.0
Dermo Es 1µM	8194.8	298.9	4819.5	233.2	3550.0	-56.5	2284.9	-52.6
Dermo Es 0,5µM	7212.8	251.1	4447.4	207.4	5335.5	-34.7	3475.2	-27.9
Dexamethasone 0,6ug/ml	3823,7	86,1	1542,8	6,6	2215,0	-72,9	1100,2	-77,2

Dermo-Es (2µM -0.5µM) inhibits IL6 and IL8 cytokines secretion, raised by LPS stimulation. The most active dose is 1µM, acting similar with Dexamethasone, and the synthetic drug – positive control. This action highlights the anti-inflammatory effect, exerted by stopping the recruitment and activation of neutrophils (IL8 inhibition), and as well as by avoiding the chronically stage of inflammation (IL6 inhibition).

Table 4: Vascular permeability analysis –VEGF factor modulated by Dermo-Es

Tested substance	**Unstimulated cells**	**LPS 1 µg/ml stimulated cells**	
	VEGF (pg/ml)	**VEGF (pg/ml)**	**% of variation**
Cellular control	10,33	46,16	
Dermo Es 2µM	64,9	31,37	-32,04
Dermo Es 1µM	46,16	21,08	-54,33
Dermo Es 0,5µM	48,2	52,3	13,3
Dexamethasone 0,6ug/ml	28,52	41,14	-10,87

Considering the inhibitory activity on the VEGF factor, correlated with reduced vascular permeability, could be assumed that Dermo-Es is an effective anti-inflammatory agent at the vascular level, with a complex and convergent mechanism of action that could be used for the inflammation trigger reducing in the acne pathogenesis.

Table 5: ICAM adhesion molecule expression: **Dermo-HdC** – comparing with cellular control and positive control

Tested substance	**Unstimulated cells**		**LPS 1µg/ml stimulated cells**	
	ICAM (median of fluorescent channel)	% of variation	ICAM (median of fluorescent channel)	% of variation compared with the unstimulated sample
Cellular control	10882.6		13974.4	28.4
Dermo-HdC 10µM	12443.3	14.3	12626.3	-9.6
Dermo-HdC 15µM	12101.1	11.1	12182.3	-12.8
Dermo-HdC 20µM	11941.2	9.7	12414.6	-11.1
Dexamethasone 0,6ug/ml	10235.8	-5.9	11097.4	-20.5

Dermo –HdC (10µM -20µM) inhibits the ICAM over-expression induced by LPS stimulation, in significant manner compared with dexamethasone, the positive control.

Table 6 : Dermo-HdC effects on extracellular release of pro-inflammatory cytokines

Tested substance	**Unstimulated cells**				**LPS 1µg/ml stimulated cells**			
	IL6 (pg/ml)	% of variation	IL8 (pg/ml)	% of variation	IL6 (pg/ml)	% of variation	IL8 (pg/ml)	% of variation
Cellular control	2054.5		1446.6		8166.1	297.5	4819.5	233.2
Dermo-HdC 10µM	7543.6	267.2	4716.8	226.1	5295.6	-35.2	3273.8	-32.1
Dermo-HdC 15µM	6188.7	201.2	4328.9	199.2	1961.2	-76	1206.2	-75
Dermo-HdC 20µM	5109.3	148.7	3185.1	120.2	4013.7	-50.8	2943.8	-38.9
Dexamethasone 0,6ug/ml	3823.7	86.1	1542.8	6.6	2215	-72.9	1100.2	-77.2

Dermo HdC (10µM -20µM) inhibits in significant manner the IL6 and IL8 discharge induced by LPS stimulation, the most active dose being 15µM, compared with the positive control anti-inflammatory action. As a consequence, Dermo-HdC acts as an anti-inflammatory drug, stopping the neutrophils recruitment and activation (IL8 inhibition), as well as avoiding the chronic stage of inflammation (IL6 inhibition).

Table 7: Vascular permeability analysis –VEGF factor modulations by Dermo –HdC

Tested substance	**Unstimulated cells**	**LPS 1 µg/ml stimulated cells**	
	VEGF (pg/ml)	VEGF (pg/ml)	% of variation
Cellular control	10.33	46.16	
Dermo-HdC 10µM	44.14	52.3	13.30
Dermo-HdC 15µM	30.42	13.88	-69.93
Dermo-HdC 20µM	19.26	30.42	-34.09
Dexamethasone 0,6ug/ml	28.52	41.14	-10.87

Dermo HdC 15 µM also confirm and strengthen the anti-inflammatory action through VEGF inhibition effective on vascular permeability, reducing the electrolyte transfer at endothelial level and minimizing edema.

I.2.. ANTI-INFLAMMATORY EFFECT – TNF-α stimulation

The experimental design mimics the systemic inflammation, VCAM expression characterizing the large vessels inflammation (edema specific), ICAM revealing the beginning of micro-vasculature inflammation (important for example in UV radiation endothelial damages)

In tables 8-13 are presented the interest values. The ICAM and VCAM expression is evaluated as median of fluorescent channel of corresponding flow-cytometry histograms, IL6, IL8 citokynes and VEGF factor are expressed in pg/ml (concentration in extracellular medium).

Table 8 : DERMO-Es comparing with cellular control and positive control: ICAM and VCAM adhesion molecules expression

Tested substance	**Unstimulated cells**				**TNF α 2ng/ml stimulated cells**			
	ICAM (median of fluorescent channel)	% of variation	VCAM (median of fluorescent channel)	% of variation	ICAM (median of fluorescent channel)	% of variation	VCAM (median of fluorescent channel)	% of variation
Cellular control	10882.6		1366.9		15697.5		1569.7	
Dermo Es 2µM	12494.9	14.8	1249.3	-8.6	16041.6	2.2	1491.1	-5.0
Dermo Es 1µM	12784.2	17.5	1348.1	-1.4	14093.3	-10.2	1157	-26.3
Dermo Es	13008.4	19.5	1314.9	-3.8	12340.5	-21.4	1344.8	-14.3

0,5µM								
Dexamethasone 0,6ug/ml	10235.8	-5.9	1339.6	-2.0	15095	-3.8	1131	-27.94

Dermo-Es 1µM is active especially on VCAM expression, similar with Dexamethasone –the anti-inflammatory drug used as positive control, but the 0.5µM dose act especially on ICAM expression. Thus, it could be recommended different doses for different target tissue (Dermo-Es 1µM for oedema- large vessels inflammation; Dermo-Es 0.5µM to reduce inflammation at micro-vasculature in photo-ageing or superficially wounds).

Table 9:Dermo-Es effects on extracellular release of pro-inflammatory cytokines

Tested substance	**Unstimulated cells**				**TNF α 2ng/ml stimulated cells**			
	IL6 (pg/ml)	% of variation	IL8 (pg/ml)	% of variation	IL6 (pg/ml)	% of variation	IL8 (pg/ml)	% of variation
Cellular control	2054.5		1446.6		8543.4	315.8	5547.2	283.5
Dermo Es 2µM	7896.8	284.4	4965.5	243.2	8900.7	4.2	5026.6	-9.4
Dermo Es 1µM	8194.8	298.9	4819.5	233.2	9113.3	6.7	5997.2	8.1
Dermo Es 0,5µM	7212.8	251.1	4447.4	207.4	6639.2	-22.3	4495.0	-19.0
Dexamethasone 0,6ug/ml	3823.7	86.1	1542.8	6.6	3218.5	-62.3	1379.9	-75.1

Dermo –Es 0.5µM acts on both IL6 and IL8 secretion on extracellular medium, stopping the inflammatory signals for neutrophils activation (including the release of enzymatic granules that degrade the connective tissue), as well as the progression from acute to chronic stage.

Table 10: Vascular permeability analysis –VEGF factor modulations by Dermo –Es

Tested substance	**Unstimulated cells**	**TNF α 2ng/ml stimulated cells**	
	VEGF (pg/ml)	VEGF pg/ml)	% of variation
Cellular control	10.33	71.35	
Dermo Es 2µM	64.9	69.19	-3.02
Dermo Es 1µM	46.16	80.08	12.23
Dermo Es 0,5µM	48.2	95.69	34.11
Dexamethasone 0,6ug/ml	28.52	48.2	-32.44

Dermo-Es is not active on the VEGF factor in terms of pro-inflammatory stimulation with TNF-α.

Table 11:ICAM and VCAM adhesion molecules expression: **Dermo-HdC** – comparing with cellular control and positive control

Tested substance	**Unstimulated cells**				**TNF α 2ng/ml stimulated cells**			
	ICAM (median of fluorescent channel)	% of variation	VCAM (median of fluorescent channel)	% of variation	ICAM (median of fluorescent channel)	% of variation	VCAM (median of fluorescent channel)	% of variation
Cellular control	10882.6		1366.9		15697.5	44.2	1569.7	14.8
Dermo-HdC 10µM	12443.2	14.3	1314.9	-3.8	13274.9	-15.4	1399.4	-10.8
Dermo-HdC 15µM	12101.1	11.2	1301.9	-4.8	13241.1	-15.6	1199.2	-23.6
Dermo-HdC 20µM	11941.1	9.7	1285.7	-5.9	12036.7	-23.3	1201.8	-23.4
Dexamethasone 0,6ug/ml	10235.8	-5.9	1339.6	-2.0	15095	-3.8	1131	-27.9

Dermo-Hdc (10µM - 20µM) inhibits ICAM and VCAM adhesion molecules in a dose depending manner.

Table 12: Dermo-HdC effects on extracellular release of pro-inflammatory cytokines

Tested substance	**Unstimulated cells**				**TNF α 2ng/ml stimulated cells**			
	IL6 (pg/ml)	% of variation	IL8 (pg/ml)	% of variation	IL6 (pg/ml)	% of variation	IL8 (pg/ml)	% of variation
Cellular control	2054.5		1446.6		8543.4	315.8	5547.2	283.5
Dermo-HdC 10µM	7543.6	267.2	4716.8	226.1	8037.9	-5.9	4971.6	-10.4
Dermo-HdC 15µM	6188.7	201.2	4328.9	199.2	7346.8	-14.0	5026.6	-9.4
Dermo-HdC 20µM	5109.3	148.7	3185.1	120.2	6262.6	-26.7	4293.5	-22.6
Dexamethasone 0,6ug/ml	3823.7	86.1	1542.8	6.6	3218.5	-62.3	1379.9	-75.1

The most active dose on IL6 and IL8 inhibition is HdC 20µM, acting on both cytokines and inducing a double effect: stopping the progression of acute inflammation (IL6 pathway) and the inhibition of neutrophil activation (IL8 signaling).

Table 13: Vascular permeability analysis –VEGF factor modulation by Dermo HdC

Tested substance	**Unstimulated cells**	**TNF α 2ng/ml stimulated cells**	
	VEGF (pg/ml)	**VEGF pg/ml)**	**% of variation**
Cellular control	10.33	71.35	
Dermo-HdC 10µM	44.14	84.5	18.43
Dermo-HdC 15µM	30.42	62.77	-12.02
Dermo-HdC 20µM	19.26	42.14	-40.93
Dexamethasone 0,6ug/ml	28.52	48.2	-32.44

Dermo HdC acts in a dose-effect manner on the VEGF factor as well, an effect that supports the anti-inflammatory activity demonstrated also on the other two cellular signaling pathways.

Based on the experimental data presented we design the following configuration concerning the anti-inflammatory action of Dermo-Es si Dermo-HdC (+ symbolizes the positive effect – stopping the inflammation on the signaling pathway investigated; - represents the absence of a significant activity) – Table 14.

Table 14. Anti-inflammatory action of Dermo-Es and Dermo-HdC

Bioactive compound/ Cellular parameter	**LPS stimulation (bacterial origin inflammation)**			**TNFα stimulation (systemic inflammation of different ethiologic**		
	Adhesion molecules (ICAM)	Pro-inflammatory cytokines IL6 siIL8	Vascular permeability (VEFG)	Adhesion molecules (ICAM si VCAM)	Pro-inflammatory cytokines IL6 siIL8	Vascular permeability (VEFG)
Dermo-Es	-	+	+	+	+	-
Dermo-HdC	-	+	+	+	+	+

Conclusions

The experimental design reveals a differentiated action of the two compounds, dependent on doses and the signaling pathway. In order to have a complete screening for the

anti-inflammatory action, it's imposed the examination of different cellular processes, convergent to the same therapeutic target. The inhibition of both IL6 and IL8 represents a synergic process blocking the neutrophils activation (including the release of enzymatic granules that degrade the connective tissue), as well as stopping the inflammation progression from acute to chronic stage. This process is accompanied by VEGF inhibition, correlated with the decrease of the vascular permeability and reducing of edema, as well as the normalization of monocyte-endothelium adhesion molecules (ICAM and VCAM) over-expression, triggered as one of the first responses on endothelial pro-inflammatory stimulation.

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

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

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

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