EVALUATION OF SOME PHYTOCOMPOUNDS EFFECTS AT HUMAN DERMAL FIBROBLAST LEVEL IN ORDER TO DEFINE THEIR DERMATOCOSMETIC ACTION

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Introduction

Effective dermatocosmetic products are used to improove the skin hydration and viscoelasticity and to maintain the normal conditions of the skin. The effect of this type of formulations may be influenced by many factors, such as type and concentration of the active substances used, as well as the composition of the vehicle. Nowadays, there are many herb's extract present in the commercial moisturizer section claiming for restoring skin hydration and viscoelasticity properties. Consequently, subjective studies to evaluate the moisturizing effect scientifically are necessary to validate these claimed effects. Objective methodologies are considered appropriate to demonstrate and to clarify the mechanisms of action of substances that improve skin properties. The effect of dermatocosmetics should be tested primarily with *in vitro* studies, complementary and correlative, in order to highlight the effect at target cells: keratinocytes, fibroblasts, vascular endothelium. Fibroblasts are the easiest cells to cultivate *in vitro*, as their growth requirements are minimal in comparison to other human cell types, being used as control cells for cell biology and inflamatory response studies, pharmacological tests and skin reconstruction. In this respect, our aim was to test the influence of some biocompounds isolated and purified from medicinal indigene flora as base for the anti-ageing products formulation on functional parameters of dermal fibroblasts and extracellular matrix.

Materials and methods

Plant material

Two specimens from medicinal indigene flora, *Salvia officinalis* and *Callendula officinalis*, were used for extraction procedures in order to obtain active biocompounds for this study. The obtained extracts were subjected to isolation and purification. Two unglycosylated compounds with β-amirinic structure were selected: Dermo-U (from *Salvia officinalis*) and Dermo-O (from *Callendula officinalis*).

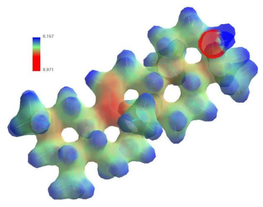


Fig.1. Aglycon 3D model of activ compounds with β-amirinic structure

Cell line

Normal human fibroblast standardized cell line HS 27 was used for *in vitro* tests. Cells were cultivated on DMEM (Dulbecco's Modified Eagle Medium) supplemented with L-glutamine and 10% fetal bovine serum.

***In vitro* screening to determine the concentration of cytotoxic effect .**

In order to determine the appropriate dose for the specific activity studies growing serial dilutions range from 2 to 100 µM were assessed in cytotoxicity studies.

LDH method

The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit from Promega was used for quantitatively measures of lactate dehydrogenase (LDH). Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay.

The manifested cellular cytotoxic effect is well correlated with cell membrane disruption and lysis. The spectrophotometric measurements were made at 490 nm with the multimode microplate reader TriStar from Berthold Technologies (Germany).

Cell viability MTS assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, is reduced to formazan in living cells, only when reductase enzymes are active, and therefore this conversion is used as a measure of viable (living) cells. The formazan concentration, which is directly proportional with viable cells number, was assessed with the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega), measuring the 490 nm absorbance (multimode microplate reader TriStar).

Investigation methods for specific *in vitro* activity determination. The following functional parameters of dermal fibroblasts were determined by flow cytometry analysis:

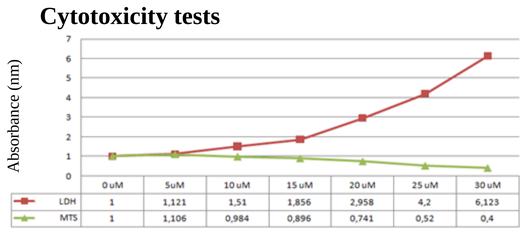
Cell proliferation and the cell cycle sequence - CellTrace™ CFSE Cell Proliferation Kit and Cycle TEST PLUS DNA Reagent kit (BD PHARMINGEN);

The intracellular calcium level - fluorescent marker FLUO-4-AM ;

Oxidative stress - DHR 123 (dihydrorhodamine) fluorescent marker.

Results and discussions

Cytotoxicity tests



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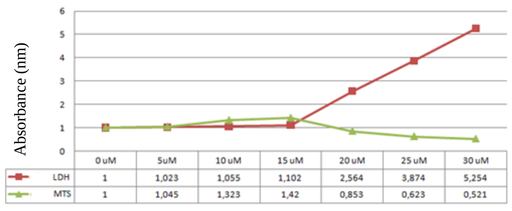
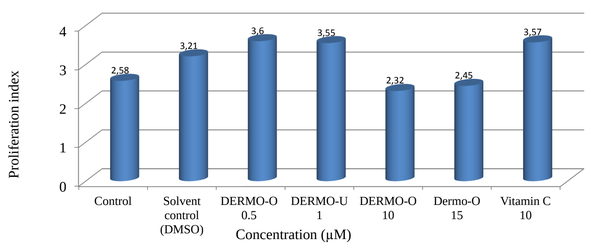


(b)

Fig.2. The Dermo U (a) and Dermo O (b) effect on metoabolic activity and LDH release

Tested dillutions allowed setting following probes concentrations for farther analysis: DERMO-U under 10 µM and DERMO-O under 20µM.

Cell proliferation and the cell cycle sequence



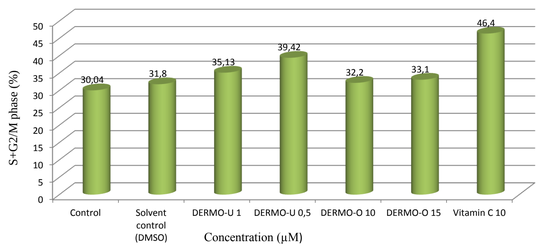
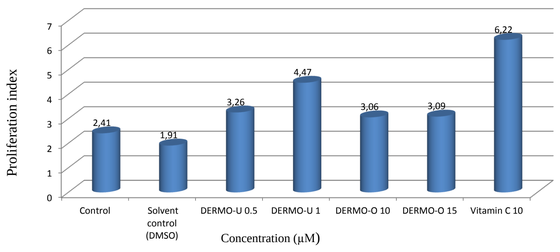


Fig. 3. Proliferation index and cell cycle S+ G2/M phase on normal human fibroblast standardized cell line HS27 (48 h adhesion and 24 h of treatment)



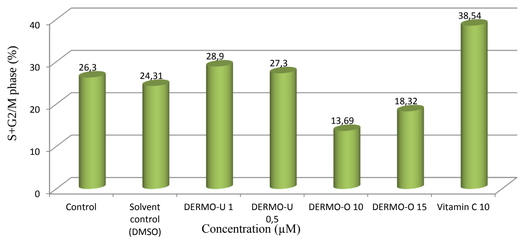
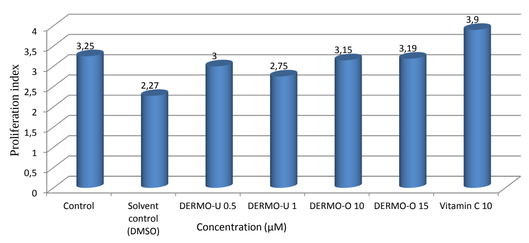


Fig. 4. Proliferation index and cell cycle S+ G2/M phase on normal human fibroblast standardized cell line HS27 (48 h adhesion and 48 h of treatment)



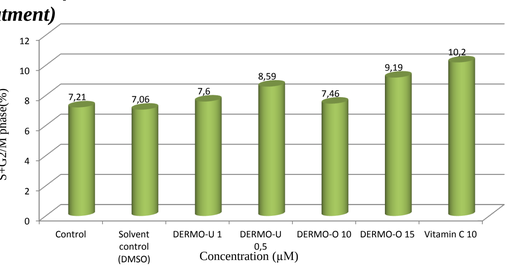
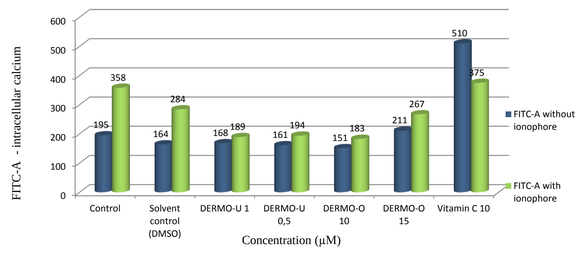


Fig. 5. Proliferation index and cell cycle S+ G2/M phase on normal human fibroblast standardized cell line HS27 (48 h adhesion and 72 h of treatment)

Cell division is more pronounced at 24h of action, the percentage of cells in DNA synthesis phase is extremely low at 72h of treatment with substances. Instead, proliferation rate increases, being higher at 72h. Increased synthesis of genetic material prepares the acceleration of proliferation. DERMO-U immediately acts on dermal fibroblasts, being active at 24 h of treatment, both on cell proliferation and the multiplication of DNA. After 72h the effect is preserved only on the proliferation index, as a consequence of the previous increased division. DERMO-O has a slower action, starting with 48h, but maintained and amplified over time. The active concentrations are 10µM respectively 15µM.

The intracellular calcium level



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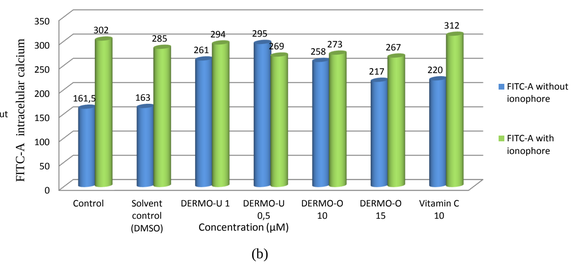
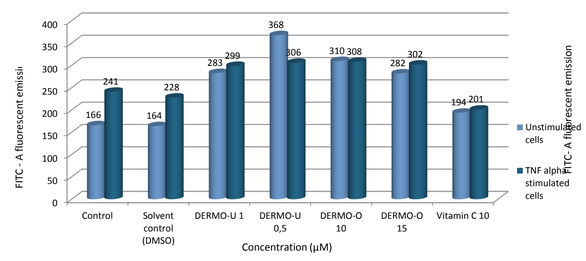


(b)

Fig. 6. Fluorescence intensity corresponding to intracellular calcium concentration, with and without ionophore, of normal human fibroblast standardized cell line HS27 at 48 h adhesion and 48 h of treatment (a) and at 48 h adhesion and 72 h (b)

Oxidative stress

Transmembrane transport activity was checked by the action of ionophore A 23187 (Calcium Ionophore) which opens the calcium channels and provides an influx of calcium ions (Ca^{2+}) from outside to inside the cell when concentration in the extracellular environment is higher then the intracytosolic one. Since oxidative stress is associated with the aging process, understanding how oxidants alter Ca^{2+} signaling can help to understand process of aging and may lead to new anti-aging strategies.



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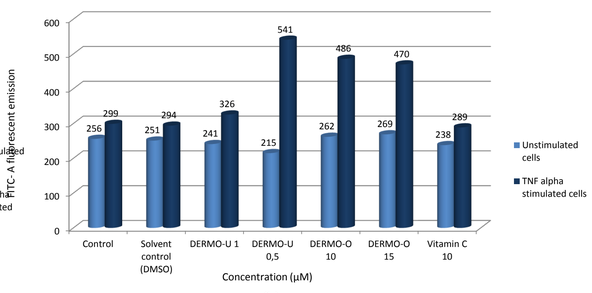

(b)

Fig. 7. Oxidative stress expressed by intracellular hydrogen peroxide release at 48 h adhesion and 48 h of treatment. (a) and 48 h adhesion and 472 h of treatment (b).

Cells were analyzed in normal development conditions (basal level of hydrogen peroxide in the presence and absence of investigated compounds) and also in an nonspecific inflammation model induced by TNF –α (pro-inflammatory citokyne) 100ng/mL, 20min, 37^0C. After 48h in the presence of tested substances has been a slight increase of basal intracellular hydrogen peroxide level associated with the activity of nonspecific cellular defense, but in terms of action of the inflammatory stimulus the next compounds manifested protection against the increased cellular oxidative stress: DERMO-U and DERMO-O 10µM. The same substances at 72h reduce basal intracellular hydrogen peroxide levels, but in pro-inflammatory conditions are no longer active.

Conclusions

In vitro tests on standardized cell lines, which relies the *in vivo* behavior of normal cells, is a priority both because of establishing the physiological mechanism and therapeutic indications as well as by fulfilling the conditions of ethics on animal testing of cosmetic products. Used techniques offer alternative methods which converge to define the effect of DERMO-U and DERMO-O.

DERMO-U acts immediately on dermal fibroblasts, being active at 24h of treatment, both on cell proliferation and the multiplication of DNA. DERMO-U has a rapid acceleration effect on fibroblasts turn-over. Active doses are in the range of 0.5 to 1µM.

DERMO-O has a slower effect on proliferation and cell cycle, acting on their stimulation only after 48h, effect maintained and even enhanced in 72h of treatment. Thus it is demontrated the delayed effect on fibroblasts growth and multiplication activity, which suggests the products combination with quick effect biocompounds (DERMO-U). DERMO-O is active in the range of 10-15µM.

Reference: Kapoor S and Saraff S, Pharmacogn Mag. 2010, 6(24):298-304; Okarinen A, Photodermatol Photoimmunol Photomed. 2000, 7(1):3-4; Tsuji N, Moriwaki S, Suzuki Y, Takema Y, Imokawa G, Photochem Photobiol. 2001, 74(2):283-90;Heinrich U, Tronnier H, Stahl W, Bejot M, Maurette J.M, Skin Pharmacol. Physiol. 2006, 19(4):224-31

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