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Original paper

Regenerative and scare healing potential of active compounds from *Camelina sativa* oil and grape pomace

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Abstract

Wound healing is a complex process that still needs improving. The keratinocyte differentiation «in vitro» model together with the evaluation of cell cycle sequentiation and collagen synthesis offer a correlative panel for a background screening of the main events that sustain the skin renewal. The experimental design was based on cytotoxicity and efficacy evaluations of keratinocytes (HaCaT cell line) and fibroblasts (HS27 cell line), through flow cytometry, microscopy and spectro-photometric techniques.

Our current study shows important effects on the aforementioned processes for two vegetal extracts that we used: *Camelina sativa* oil (CAM), a plant which was less explored until now, but with a clear potential for some therapeutical effects and the Grape pomace (TES), a waste from winery industry, used mainly on an empirical base before.

Camelina oil, through its composition rich on fatty acids, accelerates the dermo-epidermal “de novo” synthesis, stimulating the keratinocytes differentiation and turn-over and also fibroblasts cellular division and collagen synthesis. The previously described antioxidant actions of TES extract are also mainly expressed through the over-expression of involucrine, transglutaminase-1 and cytokeratine 5/14, as key events of epidermal layers growth and mithotic phases amplification in keratinocytes and fibroblasts synthesis.

All of these effects are suggesting the regenerative and scare healing potential of both extracts, with direct possible applications in pharmaceutical and cosmetic industry.

Keywords Grape pomace extract, *Camelina sativa* oil, keratinocyte differentiation, collagen, fibroblasts, skin renewal.

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Introduction

Regenerative medicine has gained new valences and an exponential development, covering areas ranging from neurological disorders (PARK & al [1]) to skin recovery after mechanical or thermal injuries. Implementing this new therapeutic concept leads to the significant rehabilitation of some cerebral disorders, as well as a decrease in the mortality rate and an increase of social re-inclusion (PAPACOCEA & al [2]). Regenerative medicine with focus on scar healing and skin renewal therapies consists of several processes that could be modulated in order to achieve effective and long-term response. Among them, we could mention coagulation, inflammation, accumulation of radical substances, proliferation, formation of fibrous tissues and collagen, contraction of wound with formation of granulation tissue and scar. After the bleeding and inflammation are controlled, the new tissue formation starts (epithelial cells and fibroblasts migrate to the damaged region, replacing dead cells and producing collagen, fibronectin, hyaluronan, glycosaminoglycans, and proteoglycans, major constituents of the extracellular matrix that assure strength to the skin (GURTNER & al [3]). The new tissue is continuously remodeled until its composition and properties are close to those of the healthy tissue (GUO & al [4]). An important goal of the wound-healing process is the regeneration of the injured skin without scar formation, which is of course hard to achieve due to the complexity of factors and inter-related signalling cascades involved. In all these stages, besides the well-known impact of oxygen radicals on molecular interactions (PAPACOCEA & al [5]), growth factors as transforming growth factor- β (TGF- β), epidermal growth factor (EGF), insulin-like growth factor-1, platelet-derived growth factor (PDGF), are key activators of fibroblasts, endothelial cells, and macrophages in the surrounding environment, facilitating signals and cell – cell or cell – protein communication. The regeneration of healthy and functional skin remains a challenge due to its multilayer structure and the presence of different cell types within the extracellular matrix in an organized way, as well as a complex panel of mediators and regulatory metabolic pathways. Thus, new insights in the combination of traditional products with modern treatments and future challenges regarding the target processes involved in skin therapies are a priority. In addition, traditional therapies based on herbal- and animal-derived compounds, living organisms, and silver and traditional dressings play an important role in all phases of the wound-healing process, allowing the treatment of a wide range of skin lesions (PEREIRA & al [6]). Natural compounds have been used since the beginnings of the medicine as phytotherapeutic agents for wound healing, and more recently purified

substances from plants have been studied proving their beneficial effect on wound treatment. For example, curcumin stimulates the production of the growth factors involved in the wound healing process, accelerating the skin restoration (TEJADA & al [7], DIECKMANN & al [8]). Current trends move to the development of innovative wound care treatments, combining the use of traditional healing agents and modern products/practices, such as nanofibers containing silver nanoparticles, Aloe vera loaded into alginate hydrogels, propolis into dressing films, and hydrogel sheets containing honey (PEREIRA & al [9], PEREIRA & al [10], ATIBA & al [11]). We could also mention traditional agents utilized for the treatment of dermatological disorders, possessing a number of pharmacological actions leading to wound healing, as in the case of *Calendula officinalis*, *Salvia officinalis*, *Hypophae rhamnoides*, etc. Current reports are demonstrating their implication on cellular and molecular targets for skin relief (TGF β , metalloproteinases, oxygen reactive species, etc) (BUTNARIU & al [12], DUMITRIU & al [13], DUMITRIU & al [14]).

Camelina oil was previously described as a good skin conditioner, especially due to its ω 3 and ω 6 fatty acids, components found also in our cold press oil (DRUMEA & al [15]). TES extract has a good skin compatibility, antioxidant and antiinflammatory effects, as our previous study also demonstrated (CRACIUN & al [16]). Considering all these arguments, we focused our studies on several mechanisms which could sustain skin regeneration, such as keratinocyte differentiation, dermal and epidermal cells turnover and collagen synthesis highlighting the **Camelina oil (CAM) and grape pomace extract (TES)** potential in the augmentation of skin renewal cycle.

Materials and Methods

1. Materials

Vegetal extracts:

- Grape pomace, the residue obtained from grapes in wine industry (**TES extract**). TES is an aqueous extract, 100 mg/ml.
- *Camelina sativa* oil (**CAM**) – obtained from ecological *Camelina sativa* seeds (the Romanian Madalina variety) through cold pressing methods

Standardized cell lines:

- **Human dermal Fibroblast** (normal cell line HS27-ATCC® CRL-1634™). Cells were cultured 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) and 1% Antibiotic Antimycotic Solution (100 \times) (Cod: A5955, Sigma Aldrich) used in these experiments between passages 32-43.

- **Normal Human Keratinocytes** (immortalized cell line HaCaT) – epithelial squamous cell with a remarkable regenerative potential, undergoing a differentiation process. Cells (passages 43-58) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (code: 30-2002, ATCC®), with high content of glucose, supplemented with 10% fetal bovine serum, 1% Antibiotic Antimycotic Solution (100×) (Cod: A5955, Sigma Aldrich).

Chemical Reagents:

- CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS), Promega
- CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega
- Cell Cycle sequencing (BD Cycletest Plus DNA kit - 340242);
- Cytokeratin 5/14PE – Santa Cruz Biotechnology, SC58733; TGase 1 (A-5), mouse monoclonal IgG – Santa Cruz Biotechnology, SC365821 – primary antibody and Anti- mouse IgG – FITC – Sigma F2883 as secondary antibody; involucrin (H-120) Santa Cruz Biotechnology, SC28557 rabbit polyclonal IgG primary antibody and goat Anti-rabbit IgG-R, rhodamine conjugate Santa Cruz Biotechnology, SC2091 as secondary antibody.

2. Methods

a) **Cell cytotoxicity** – cell viability monitoring (MTS test), correlated with the estimation of the sample toxicological impact (release of lactate dehydrogenase – LDH assay) (FOTAKIS & al [17], RISS & al [18]).

b) **DNA synthesis phase** evaluation from cell cycle enables identification of DNA replication cycle, applying nuclei fluorescence labeling with propidium iodide, followed by flow-cytometric quantification.

c) **Keratinocytes Differentiation:** Specific fluorescent antibodies staining for cytokeratine 5/14, transglutaminase 1 and involucrine membrane expression and flow cytometry quantitation.

d) **Collagen quantification (Sirius red and Fast Green)** Cells fixed with Methanol at -20°C, 20 min are coloured with Sirius red/Fast green (Chondrex, Gentaur) for 30 min, at room temperature. After incubation with colorants, cells are rinsed with 0.01N HCl and visualised at optical microscope. For a total collagen quantification, the dye reacted with collagen was eluted with 1 ml. NaOH 0.1N, and the absorbance quantified at 540 nm (for collagen/Sirius Red) and 605 nm (non-collagen proteins / Fast Green).

Equipments and software:

- FACS CANTO II – flow cytometer (Becton – Dickinson), with DIVA 6.1 software for differentiation process quantification and FCS Express – Multicycle AV (Phoenix flow systems) software for cell cycle analysis;
- Optical microscope Nikon; Plate Reader Berthold technologies;
- UV/VIS Spectrometer, Lambda 25, Perkin Elmer.

Statistics: Data are expressed as mean ± standard deviation (SD), averaged over at least three independent experiments for normally distributed data. We consider *P< 0.05, ** P< 0.01, ***P< 0.001, ****P< 0.0001, using Repeated Measures ANOVA, Dunnett's Multiple Comparison Test.

Results and Discussion

CYTOTOXICITY of Camelina oil (CAM)

Camelina oil was solubilised in DMSO, 20% stock solution. Starting from 0.2% concentration, the camelina oil has no cytotoxic effect on keratinocytes and fibroblasts, after 48 h of incubation. According with the graphic (Fig. 1 and 2), the Camelina oil preserve the metabolic activity as the same level than the cellular control, with no significant release of lactate dehydrogenase in the culture medium.

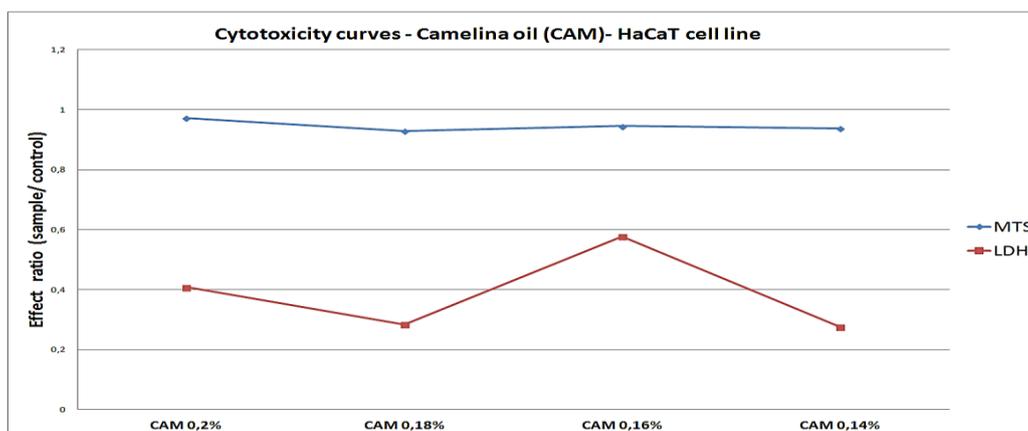


Figure 1. Evaluation of Camelina oil cytotoxicity on HaCaT cell line

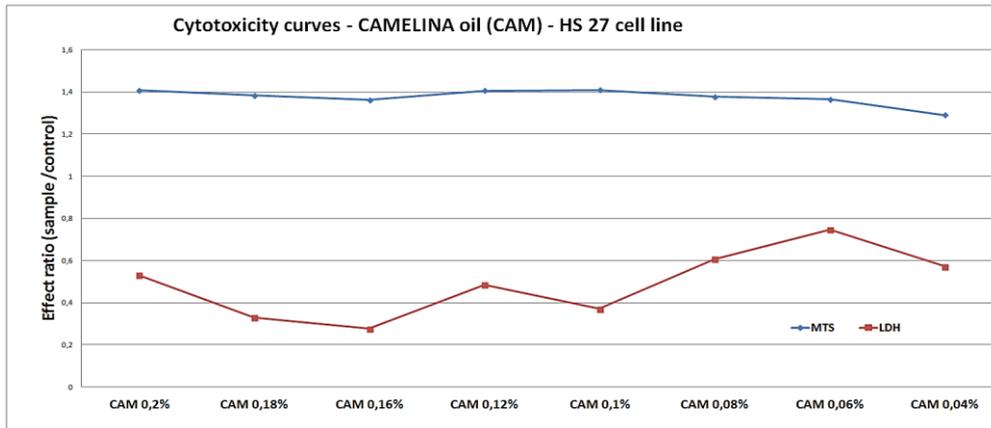


Figure 2. Evaluation of Camelina oil cytotoxicity on HS27 cell line

Skin regeneration potential of both vegetal extracts was investigated through the following mechanisms:

- **Epithelization**, as an essential component of wound healing, used as a defining parameter of a successful wound closure – *in vitro* transposed through keratinocytes differentiation (epidermal layer highlight: stratum basal – cytokeratine 5/14 expression, stratum spinosum – transglutaminase-1 and stratum corneum – involucrine membranar expression), correlated with the stimulation of cellular division leading to an accelerated turn-over.
- **Dermal restoring** through fibroblasts cell cycle acceleration and collagen synthesis amplification.

The evaluation of keratinocyte differentiation process was done through specific protein expression (IRVINE & al [19]), as following: Involucrine (stratum corneum and granulosum), Transglutaminase (stratum spinosum), keratine K 5/14 (stratum basal). The keratinocytes differentiation is „in vitro” regulated by external calcium concentration. For example, keratinocytes in 1.2mM and 2.4mM synthesize involucrine and transglutaminase before being confluent, assuring the cornified shell at the confluence (BIKLE & al [20], BIKLE [21]).

We configure three experimental series with keratinocytes in culture medium with different calcium concentration, teated with vegetal extracts for 5 days:

- Culture medium without calcium: DMEM without calcium and magnesium, – Gibco 21068-028, supplemented with sodium pyruvate 1mM, 4mM glutamine, 10% SFB and 1% antibiotic / antimycotic
- Culture medium with **6mg Ca²⁺**: DMEM without calcium and magnesium, – Gibco 21068-028, supplemented with sodium pyruvate 1mM, 4mM glutamine, 10% SFB and 1% antibiotic / antimycotic; calcium chloride up to **6mg Ca²⁺**
- Culture medium with **12mg Ca²⁺**: DMEM without calcium and magnesium, – Gibco 21068-028, supplemented with sodium pyruvate 1mM, 4mM glutamine, 10% SFB and 1% antibiotic / antimycotic; calcium chloride up to **12mg Ca²⁺**

The keratinocytes differentiation stages were highlighted through staining with specific antibodies, as previously described, and visualised by flow cytometry. (Fig. 3).

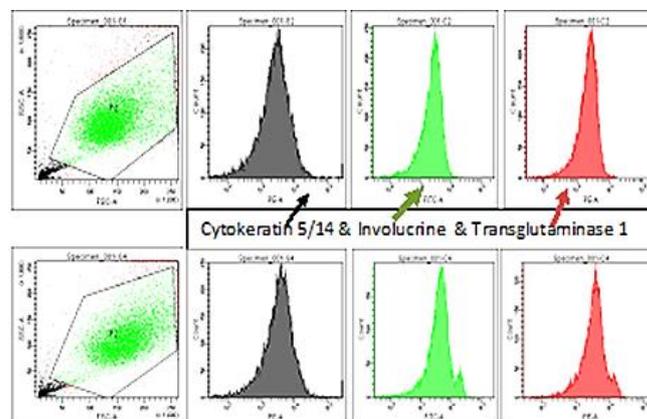


Figure 3. Flow cytometry evaluation of keratinocytes population (green dot plot) and fluorescence histograms corresponding to cytokeatine 5/14, involucrine and transglutaminase-1.

Results concerning the keratinocytes turn-over (cell cycle phases) and differentiation markers expression are presented in the Table 1 and 2.

Camelina oil and TES 2 mg/ml stimulated in a significant manner the cytokeratine 5/14 expression even in lack of Calcium in culture medium, suggesting an epidermal restoration that began from stratum basal. Both extracts also over express transglutaminase -1 and

involucrine, assuring a deeply renewal cycle of the epidermis. Results are sustained by mitotic phases augmentation, especially in already differentiated cells (culture medium with high calcium concentration).

Dermal restoring was evaluated by fibroblasts cellular dynamics stimulation and collagen synthesis amplification. The modulation of these mechanisms by the vegetal extracts TES and CAM is summarised in Table 3.

Table 1. Keratinocytes differentiation markers evolution and vegetal extracts impact (*P< 0.05, ** P< 0.01, ***P< 0.001, ****P< 0.0001, using Repeated Measures ANOVA, Dunnett's Multiple Comparison Test)

HaCaT - 5 days incubation with extracts	Involucrine (fluorescence peak channel) – STRATUM CORNEUM			Transglutaminase 1 (fluorescence peak channel) – STRATUM SPINOSUM			Cytokeratine 5/14(fluorescence peak channel) – STRATUM BASALE		
	Without calcium	6 mg calcium	12 mg calcium	Without calcium	6 mg calcium	12 mg calcium	Without calcium	6 mg calcium	12 mg calcium
Control cells	901.0 ± 23.18	1063 ± 39.21	1394 ± 8.185	776.3 ± 35.73 ^{ns}	759.0 ± 27.84	786.7 ± 40.61	1073 ± 32.79	1032 ± 26.08	1261 ± 42.67
TES 0.5mg/ml	925.7 ± 22.28 ^{ns}	998.7 ± 100.6 ^{ns}	1426 ± 28.31 ^{ns}	745.0 ± 52.03 ^{ns}	765.0 ± 30.51 ^{ns}	829.7 ± 19.86 ^{ns}	1119 ± 27.32*	989.7 ± 20.50 ^{ns}	1276 ± 62.75 ^{ns}
TES 1mg/ml	944.3 ± 20.82 ^{ns}	1090 ± 135.4 ^{ns}	1548 ± 17.78**	803.3 ± 61.04 ^{ns}	796.3 ± 43.66 ^{ns}	869.0 ± 36.50*	1122 ± 33.15*	1066 ± 78.08 ^{ns}	1322 ± 38.00*
TES 2mg/ml	1118 ± 24.99***	1301 ± 65.34**	1813 ± 80.88***	852.0 ± 51.03	996.7 ± 46.50***	1894 ± 87.71***	1134 ± 52.89**	1401 ± 10.50***	1572 ± 37.29***
CAM 0.04%	1395 ± 9.074***	1190 ± 42.55 ^{ns}	3273 ± 46.36***	1117 ± 88.64***	946.0 ± 36.76***	1556 ± 38.28***	1394 ± 28.10***	1062 ± 39.21 ^{ns}	1754 ± 34.00***
CAM 0.02%	1277 ± 23.029***	1049 ± 26.95 ^{ns}	1374 ± 34.43 ^{ns}	1065 ± 52.21***	855.7 ± 28.59*	1075 ± 51.50***	1308 ± 19.14***	1158 ± 50.66**	1293 ± 23.63 ^{ns}

Table 2. Vegetal extracts modulation of keratinocytes turn-over in differentiation conditions (*P< 0.05, ** P< 0.01, ***P< 0.001, ****P< 0.0001, using Repeated Measures ANOVA, Dunnett's Multiple Comparison Test)

Mithotic phases of HaCaT cells in differentiation conditions (5 days incubation)	Mithotic phases of keratinocytes cell cycle (% S+%G2/M)		
	Without calcium	6mg calcium	12 mg calcium
Martor	15.64 ± 0.8433	20.12 ± 0.5059	7.63 ± 0.9309
TES 0.5 mg/ml	17.40 ± 0.9450 ^{ns}	20.51 ± 1.286 ^{ns}	11.85 ± 0.9406 ^{ns}
TES 1 mg/ml	18.74 ± 1.1990*	21.76 ± 1.302 ^{ns}	13.54 ± 0.8058*
TES 2 mg/ml	19.66 ± 1.7960**	22.28 ± 2.968 ^{ns}	16.59 ± 3.215***
CAM 0.04%	20.24 ± 2.356**	23.48 ± 3.177 ^{ns}	17.58 ± 2.255***
CAM 0.02%	17.98 ± 1.054 ^{ns}	21.18 ± 3.078 ^{ns}	12.39 ± 0.8083*

Table 3. TES and CAM extracts influence on fibroblast cell cycle

	Mithotic phases of fibroblasts cell cycle (% S+%G2/M)
Control cells	37.53 ± 1.340
TES 0.5mg/ml	39.88 ± 0.6322 ^{ns}
TES 1mg/ml	43.17 ± 1.463***
TES 2mg/ml	48.55 ± 1.232***
CAM 0.04%	47.423 ± 1.274***
CAM 0.02%	42.57 ± 1.206**

Both TES and CAM are significantly active in stimulating the fibroblasts cell cycle S and G2/M phases, corresponding to DNA duplication and enter in mitosis, expanding the cells division rate and leading to an accelerated turn-over.

Total collagen detection – Sirius Red/Fast Green staining

Fibroblasts (HS27cell line) were harvested after 24 h of adhesion/ 48 h and 72 h incubation with vegetal extracts. After staining with Sirius Red / Fast Green it could be noticed pink fibrils of collagen and green proteins from extracellular matrix (Fig. 4).

After the spectrophotometric quantification of total collagen from extracellular matrix (Fig. 5, (a.)), it could be noticed the rise of collagen synthesized by fibroblasts treated with camelina oil as following: after 48 hours of treatment, in samples treated with CAM 0.08%, a 22% increase in collagen synthesis was observed compared to the solvent control. The same trend was observed after 72 hours of treatment but with only 11 % increase of biosynthesized collagen.

TES extract stimulates the intracellular synthesis of collagen in a dose effect manner so that at the highest dose applied – 6 mg/ ml is observed a noticeable increase in the amount of collagen both after 48 hours (15%) and after 72 hours (27%) of treatment (Fig. 5, (b.)).

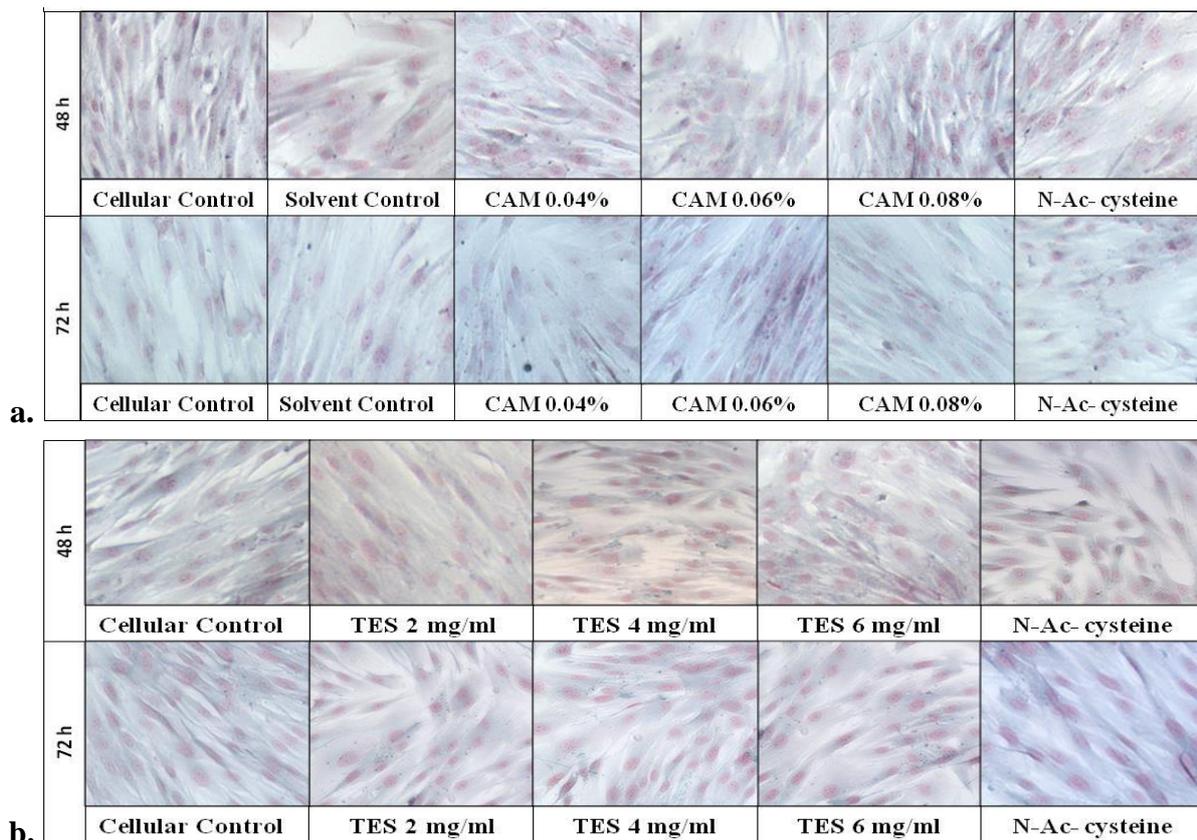


Figure 4. Total collagen and proteins from extracellular matrix detection with Sirius Red/Fast Green staining after 48 h / 72 h treatment with CAM extract (a) and TES extract (b).

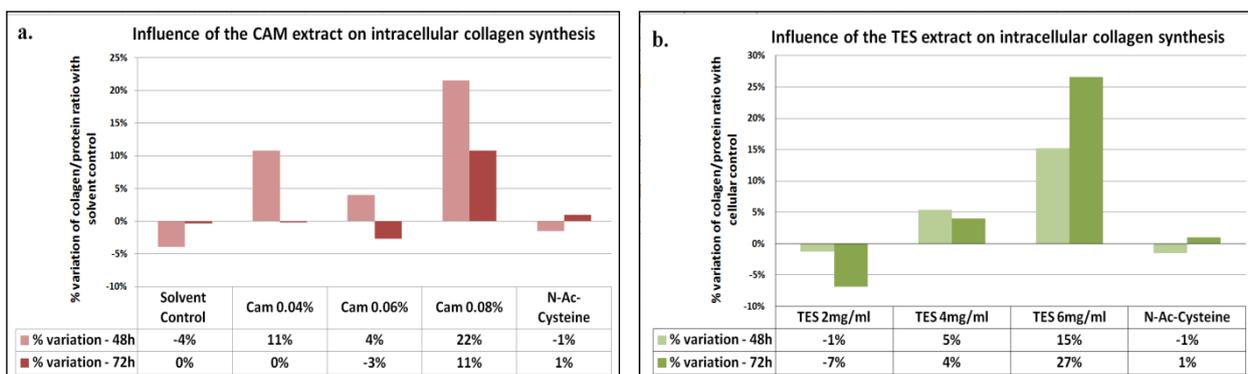


Figure 5. Total collagen / non-collagen proteins quantification (percentage modification) from extracellular matrix of fibroblasts treated with CAM (a) and TES (b).

Conclusions

The keratinocyte differentiation « in vitro » model together with the evaluation of cell cycle sequentiation and collagen synthesis offer a correlative panel for a background screening of the main processes that sustain the skin renewal.

Our current data are showing important effects on the aforementioned processes for two vegetal extracts: *Camelina sativa* oil (CAM), a plant which was less explored until now, but with a clear potential for some therapeutical effects and the Grape pomace (TES), a waste from winery industry, used mainly on an empirical base before.

Camelina oil, through its composition rich on fatty acids, accelerates the dermo-epidermal “de novo” synthesis, stimulating the keratinocytes differentiation and turn-over and also fibroblasts cellular division and collagen synthesis.

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All of the afomentioned effects are suggesting the regenerative and scare healing potential of both extracts, with direct possible applications in pharmaceutical and cosmetic industry.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

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