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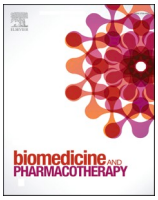

Optimization of human skin keratinocyte culture protocols using bioactive molecules derived from olive oil

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ABSTRACT

Skin damage due to severe burns can compromise patient life. Current tissue engineering methods allow the generation of human skin substitutes for clinical use. However, this process is time-consuming, as the keratinocytes required to generate artificial skin have a low proliferation rate in culture. In this study, we evaluated the pro-proliferative effects of three natural biomolecules isolated from olive oil: phenolic extract (PE), DL-3,4-dihydroxyphenyl glycol (DHFG), and oleuropein (OLP), on cultured human skin keratinocytes. The results showed that PE and OLP increased the proliferation of immortalized human skin keratinocytes, especially at concentrations of 10 and 5 μg/mL, respectively, without altering cell viability. In contrast, DHFG did not produce a significant improvement in keratinocyte proliferation. In normal human skin keratinocytes obtained from skin biopsies, we found that PE, but not OLP, could increase the number of keratinocyte colonies and the area occupied by these cells. Furthermore, this effect was associated with increased *KI-67* and Proliferating cell nuclear antigen (*PCNA)* gene expression. Thus, we propose that PE positively affects keratinocyte proliferation and could be used in culture protocols to improve bioartificial skin generation by tissue engineering.

1. Introduction

Human skin plays an important role in protecting the body against external agents and regulating body temperature. However, the skin can be affected by multiple factors that may compromise the integrity of this organ [1,2]. Skin burns, especially extensive full-thickness burns, are critical conditions representing a therapeutic challenge because the regenerative capacity of the native skin is severely compromised [3,4]. The gold-standard treatment for severely burnt patients involves covering the damaged areas using autografts obtained from unaffected skin areas. However, this method has several limitations, including the lack of undamaged areas in patients and donor-site morbidity [5,6]. For this reason, new therapeutic alternatives are needed.

Bioartificial skin substitutes generated in the laboratory by tissue engineering (TE) represent novel therapeutic options for patients with severe burns [7]. The UGRSKIN is a skin model currently being applied to patients with severe burns and has been developed by our research group [8]. Although UGRSKIN is a viable alternative for the treatment of patients with severe burns [8], some limitations associated with the biofrabication process must be resolved. An abundant population of cultured skin keratinocytes is required to generate an efficient skin substitute that can cover extensive burns [9,10]. However, human

Abbreviations: BGN, biglycan; KRT1, cytokeratin 1; KRT3, cytokeratin 3; KRT8, cytokeratin 8; KRT10, cytokeratin 10; KRT18, cytokeratin 18; KRT19, cytokeratin 19; DHFG, DL-3,4-dihydroxyphenyl glycol; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; FLG, filaggrin; GJA4, gap junction protein alpha 4; JUP, junction plakoglobin; KC medium, Keratinocyte culture medium; MKI67, marker of Proliferation KI67; NEG, negative control group; HNSK, normal human skin keratinocytes; OLP, oleuropein; PE, phenolic extract; PBS, phosphate-buffered saline; PKP1, plakophilin 1; CTR, positive control group; PCNA, proliferating cell nuclear antigen; TE, tissue engineering.

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keratinocytes typically show a slow proliferation rate and tend to dedifferentiate in culture, making this process time-consuming for patients with life-threatening conditions [11–13]. Therefore, new protocols that can improve human keratinocyte culturing methods must be developed.

A possible method to improve keratinocyte expansion is using conditioned culture systems enriched with bioactive molecules. Several natural compounds have been used for this purpose, including Spirulina extract, gentisic acid, curcumin, and Brazilian propolis, which have demonstrated the ability to enhance keratinocyte proliferation [14–17].

A promising source of bioactive molecules is olive oil from *Olea europaea*, which has demonstrated numerous beneficial anti-inflammatory, antimicrobial, and antioxidant effects [18–20]. The main bioactive compounds found in olive oil are maslinic acid, DL-3, 4-dihydroxyphenyl glycol (DHFG), oleuropein (OLP) and phenolic extract (PE) containing high concentrations of 3-hydroxytyrosol. These compounds showed beneficial effects to improve wound healing, and are thought to improve skin regeneration in vivo [21]. In addition, some of these bioactive compounds were previously tested in cell cultures, including 3-hydroxytyrosol, which was demonstrated to improve keratinocyte re-epithelialization by activating cyclin-dependent kinase 2 and 8 and metalloproteinase-9 [22], and maslinic acid, which was able to induce keratinocyte proliferation [23]. However, the potential usefulness of DHFG, OLP and PE to improve *ex vivo* human skin keratinocyte proliferation, and therefore, their usefulness in skin tissue engineering has not been determined.

In the present study, we aimed to determine the biological effects of a phenolic extract (PE) and two bioactive molecules (DHFG and OLP), derived from olive oil, on human keratinocyte cell cultures for further application in TE protocols.

2. Materials and methods

2.1. Immortalized keratinocyte cell cultures

To determine the adequate conditions required to improve human keratinocyte proliferation without affecting cell viability, we first evaluated the effect of a wide range of PE, DHFG, and OLP concentrations on immortalized normal human skin keratinocytes (Ker-CT or CRL-4048 cell line). These cells were derived from human foreskin and were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in KC (keratinocyte culture) medium [24] containing a 3:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Nutrient Mixture Ham F-12 supplemented with 10% fetal bovine serum (FBS), 1% antibiotics/antimycotics, 24 μg/mL adenine, 0.4 μg/mL hydrocortisone, 5 μg/mL insulin, 10 ng/μL epidermal growth factor and 1.3 ng/mL triiodothyronine (all products from Merck, Burlington, MA) at 37 °C and 5% CO_2. KC medium was replaced every three days, and cells were dissociated before reaching confluence using CTS TrypLE Select Enzyme solution (Invitrogen, Waltham, MA).

In this study, the following groups were considered: 1) human keratinocyte cells cultured in KC medium (positive control group of live cells or CTR), 2) cells subjected to incubation with 2% Triton X-100 (Probus, Barcelona, Spain) (negative control group of dead cells or NEG), 3) cells incubated in KC medium supplemented with 1, 5, 10, 20, 40, or 80 μg/mL of the bioactive OLP: OLP1, OLP5, OLP10, OLP20, OLP40, and OLP80, respectively (Merck, ref. 4,12247) cells incubated in KC medium supplemented with 1, 5, 10, 20, 40, or 80 μg/mL of DHFG: DHFG1, DHFG5, DHFG10, DHFG20, DHFG40, and DHFG80, respectively (Merck, ref. D9753), 5) cells incubated in KC medium supplemented with 1, 5, 10, 20, 40, or 80 μg/mL of PE: PE1, PE5, PE10, PE20, PE40, and PE80, respectively (from Extractos y Derivados SL, Granada, Spain), and 6) cells incubated in a mixture of the conditions showing the adequate results in the previous groups. In all cases, cells were cultured in 96 well-plates at a density of 7.25×10^3 cells per well and kept in KC medium for 24 h to allow cell attachment. Then, this medium was replaced by each specific culture medium, and cells were kept for 24, 48, and 72 h in a cell incubator at 37 ºC.

2.2. Analysis of cell viability

To determine the effects of the treatments analyzed in this study on cell viability, we conducted two analyses for each study group. First, cells were analyzed using the LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen, Waltham, MA) following the instructions provided by the manufacturer. In brief, cells were washed twice in phosphate-buffered saline (PBS) (Merck, Darmstadt, Germany) and incubated in darkness for 5 min in the LIVE/DEAD reagent. Samples were then washed in PBS, and fluorescence microscopy images were taken using a ZOE Fluorescent Cell Imager (Bio-Rad, Hercules, CA, USA). In each sample, the number of viable cells (stained in green) and dead cells (stained in red) were quantified, and the percentage of live cells was determined. Second, the amount of DNA released by disrupted cells to the culture medium was quantified in each sample type. In short, the supernatant medium in which cells were cultured was collected after 24, 48, and 72 h of culture, and DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Results were normalized to the positive control group (considered as 100% viability) and the negative control group (considered as 0% viability) as previously reported [23]. All analyses were carried out in sextuplicates ($n = 6$).

2.3. Analysis of cell proliferation

To assess the pro-proliferative effects of each treatment on human keratinocytes, we used a double analysis approach that included counting the number of cells and analyzing their proliferative status using a metabolic assay. In the first analysis, keratinocytes corresponding to each treated group and each follow-up time were washed in PBS and detached from the culture surface using CTS TrypLE Select Enzyme solution for 5 min at 37 °C. Cells were harvested, and the detachment solution was inactivated using 10% FBS. This solution, containing the detached cells, was then analyzed using a NovoCyte flow cytometer (Acea Biosciences/Agilent Technologies, Santa Clara, CA) to quantify the total number of keratinocytes found under each culture condition. In the second analysis, cells were analyzed using the metabolic assay Cell Proliferation Reagent WST-1 (Roche, Basel, Switzerland). Briefly, the WST-1 reagent was dissolved in KC medium and added to each well. After 4 h of incubation at 37 °C in darkness, the absorbance of the formazan dye, which correlates with the proliferative status of the cells, was measured using an Asys UVM-340 scanning microplate reader (Biochrom/Harvard Bioscience, Holliston, MA). For cell quantification and WST-1 assay, results were normalized to the positive control group (considered as 100% viability) and negative control group (considered as 0% viability) as previously reported (Ortiz-Arrabal et al., 2022). All analyses were carried out in sextuplicates ($n = 6$).

2.4. Proliferative effects on primary cultures of normal human native skin keratinocytes (HNSK)

Once we determined the specific conditions that could successfully promote cell proliferation in immortalized human skin keratinocytes derived from the human foreskin, we evaluated the proliferative effects of these conditions on primary cultures of normal human skin keratinocytes (HNSK) established from human foreskin biopsies. Human foreskin samples were obtained from three healthy patients subjected to circumcision.

To generate primary cultures of HNSK, we used the method originally described by Rheinwald and Green [25]. First, a feeder layer of 3T3 murine embryonic fibroblasts was cultured in 6-well plates using DMEM supplemented with 10% FBS. When cultures reached subconfluence,

cells were inactivated with 10 μg/mL mitomycin C (Merck) for 2 h at 37 ºC, followed by several thorough washes in PBS to remove all remaining mitomycin C. Then, the foreskin biopsies obtained from the three donors were washed in PBS and normal keratinocytes were detached by incubating the samples in a solution consisting of 0.5 g/L trypsin and 0.2 g/L ethylenediaminetetraacetic acid (Merck) at 37 ºC for 30 min with slight agitation. The supernatant was collected, the detaching solution was inactivated with 10% FBS, and the cells were harvested by centrifugation at 1000 rpm to obtain a cell pellet. This pellet was resuspended in a small volume of KC medium and kept at 37 °C. This process was repeated six times for the remaining tissue, and all the cell pellets were collected in a single tube containing a keratinocyte cell suspension. Finally, this suspension was evenly distributed on the surface of the previously inactivated 3T3 cultures and incubated for 28 days in a cell incubator using KC medium (control group) or medium enriched with the different treatments and concentrations that showed positive results on immortalized keratinocytes. Each culture medium was renewed every three days. All analyses were performed for 15 replicates per group (n = 15). Brightfield microscopy images were obtained from each culture well after 7, 14, 21, and 28 days of follow-up. The number of keratinocyte colonies found in each well was quantified at each follow-up time to determine the average number of colonies per time and condition. At the end of the follow-up period (day 28th of culture), cells were fixed in 4% formaldehyde (Panreac, Barcelona, Spain) and stained with hematoxylin-eosin to quantify the total area of each well that was occupied by keratinocyte colonies.

2.5. Analysis of HNSK gene expression by real-time quantitative PCR (RT-qPCR)

To determine the effects of the different treatments on normal human keratinocyte gene expression, we carried out RT-qPCR assays using the specific conditions that showed the most adequate results in terms of cell viability and cell proliferation (5 μg/mL of OLP and 10 μg/mL of PE) and controls (cells cultured in KC). Six samples were used for each study group (n = 6). HNSK cells were cultured for 28 days, as described above, and total RNA was extracted from each culture using a Qiagen RNeasy mini kit (Qiagen, Venlo, Netherlands). Then, 1 μg of RNA from each sample was retrotranscribed to cDNA using an iScript Advanced cDNA Synthesis kit (Bio-Rad, Hercules, CA). For gene expression analysis, custom-made PRIME-PCR plates (Bio-Rad) were used. Each plate contained primers for the quantitative evaluation of 30 different genes related to cell proliferation (Marker of Proliferation KI67 [*MKI67*] and Proliferating cell nuclear antigen [*PCNA*]), cytokeratin expression *(KRT1, KRT3, KRT5, KRT6A, KRT7, KRT8, KRT10, KRT13, KRT18*, and *KRT19*), cell attachment and intercellular junctions *(FN1, DSP, PKP1, PPL, JUP, TJP1, TJP2, GJA1, GJA2,* and *GAJ4*), epithelial differentiation (*BGN, FLG*, and *IVL*), synthesis of basement membrane components (*COL4A1, LAMA1, LAMA3, LAMB1*, and *LAMC1*), and a control housekeeping gene (*GAPDH*). For each sample, 1 μL of cDNA was mixed with 9 μL of nuclease-free H2O and 10 μL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and added to each well of the plate as indicated by the manufacturer. Then, a thermocycling protocol with an annealing temperature of 60 °C was conducted using a CFX Connect Real-Time Detection System (Bio-Rad). RT-qPCR results were normalized to GAPDH (housekeeping gene) expression for all conditions using the CFX Manager 3.1 software. Finally, the ΔΔCt value was calculated for each gene and sample.

2.6. Statistical analysis

All results obtained for each study group and each analytical method were shown as averages and standard deviations and as medians and quartile ranges (first and third quartile). To compare the results among the different study groups, we first analyzed each distribution using the Shapiro–Wilk test to determine whether these variables follow a normal distribution. Because most distributions were not normal, we used non-parametric statistics.

On the one hand, statistical comparisons were carried out using two-way ANOVA testing for non-parametric distributions. This statistical test was used to quantify the effects of two variables on the analysis results (for example, the concentration of a specific compound and the culture time), as well as the interaction between both variables. On the other hand, one-way Aligned Rank Transform (ART) ANOVA. This test allowed us to globally determine the presence of statistical differences among several study groups considered altogether (for example, all concentrations of a specific compound at a specific culture time). These tests were applied to the results of the cell viability and cell proliferation analyses, and for the quantification of the pro-proliferative effects on primary cultures of human native skin keratinocytes. Then, specific comparisons between two individual study groups were performed using pairwise testing with the Mann–Whitney exact test. Comparison of the areas occupied by keratinocyte colonies was conducted using Fisher's exact test, and the results were expressed as percentages.

For the RT-qPCR results, we first determined the fold-change in relative expression of each experimental group compared with that in the CTR group (considered as 1). The statistical significance of the differences between each experimental group and CTR was evaluated using the Mann–Whitney exact test. Genes showing a significant difference and a positive fold-change were considered upregulated, whereas significant genes with a negative fold-change were considered downregulated.

Statistical analyses were performed using the Real Statistics software for Excel, freely available at http://www.real-statistics.com (Dr. Charles Zaiontz, Purdue University, West Lafayette, IN, USA). The significance level was set at 5% for all tests.

3. Results

3.1. Cell viability of human keratinocytes cultured with PE, DHFG or OLP

The cytotoxic effects on human skin keratinocytes cultured in the presence of PE, DHFG, or OLP were assessed using two different methods. On the one hand, cell viability was measured with LIVE/DEAD kits. As shown in Figs. 1–3 and in Supplementary Tables S1-S4, cell viability was high for most study groups. When results were analyzed using two-way ANOVA testing, we found some differences among compounds. For PE, a significant association was found between LIVE/DEAD results and the concentration ($p < 0.0001$) and the culture time ($p < 0.0001$). However, the analysis showed that the interaction between concentration and culture time was not statistically significant ($p = 0.9872$). Similar results were found for DHFG ($p < 0.0001$ for the concentration, $p = 0.0031$ for the culture time and $p = 0.9812$ for the interaction) and for OLP ($p < 0.0001$ for the concentration, $p < 0.0001$ for the culture time and $p = 0.9984$ for the interaction). When results were globally compared using ART-ANOVA, we found statistically significant differences among all study groups for PE, DHFG, or OLP. Specifically, the pairwise analysis showed some differences with the CTR group. As compared to CTR, our results showed a statistically significant decrease in cell viability at 24, 48, and 72 h for the highest concentration (80 μg/mL) of PE ($p = 0.0260$, $p = 0.0022$, and $p = 0.0022$, respectively) and DHFG ($p = 0.0087$, $p = 0.0022$, and $p = 0.0022$, respectively) (Fig. 1A, B, 2A, and B and Supplementary Tables S1–S3). Additionally, as compared to the CTR group, no changes in viability were found in cells cultured with OLP ($p > 0.05$) (Fig. 3A and B and Supplementary Table S4). As expected, all experimental conditions were significantly different from NEG control. Specific differences among groups are described in Supplementary Tables S1–S4.

On the other hand, quantification of DNA released to the media by dead cells showed that cell viability was very high (above 90%) for all concentrations and culture times (Figs. 1–3 and Supplementary Table S1). Analysis of DNA release using two-way ANOVA revealed a

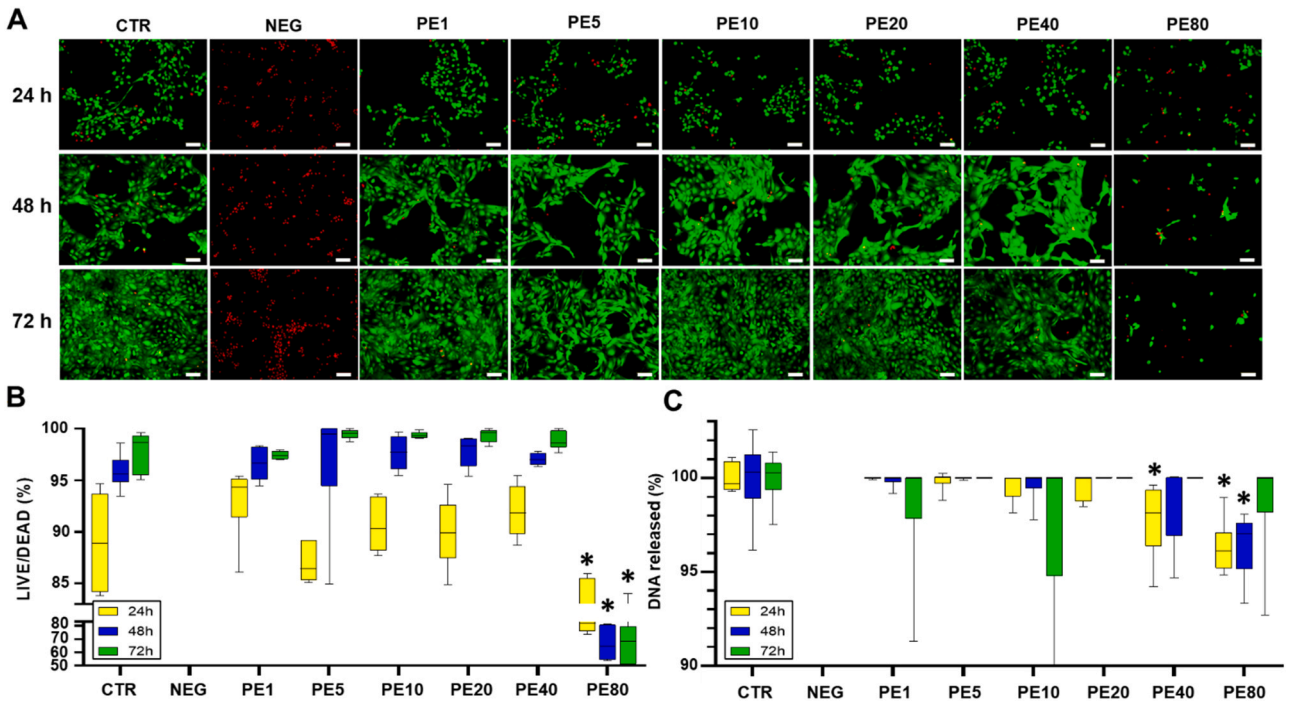

Fig. 1. Analysis of cell viability in human skin keratinocytes cultured with different concentrations of phenolic extract (PE) (1, 5, 10, 20, 40 and 80 μg/mL) for 24, 48 and 72 h. Cells cultured in basal medium without PE were considered as CTR. As negative control of cell death (NEG), cells were treated with 2% triton X-100. **(A)** Fluorescence microscopy images of cells incubated with LIVE/DEAD system. Live cells are stained in green and dead cells are stained in red. Scale bar: 100 μm. **(B)** Box and Whisker plot representation of quantification of live and dead cells treated with LIVE/DEAD kit. **(C)** Box and Whisker plot of quantification of DNA released to the medium by dead cells. In **(C)**, values were normalized regarding CTR group. Asterisks (*) refer to values with statistically significant differences with respect to the CTR group.

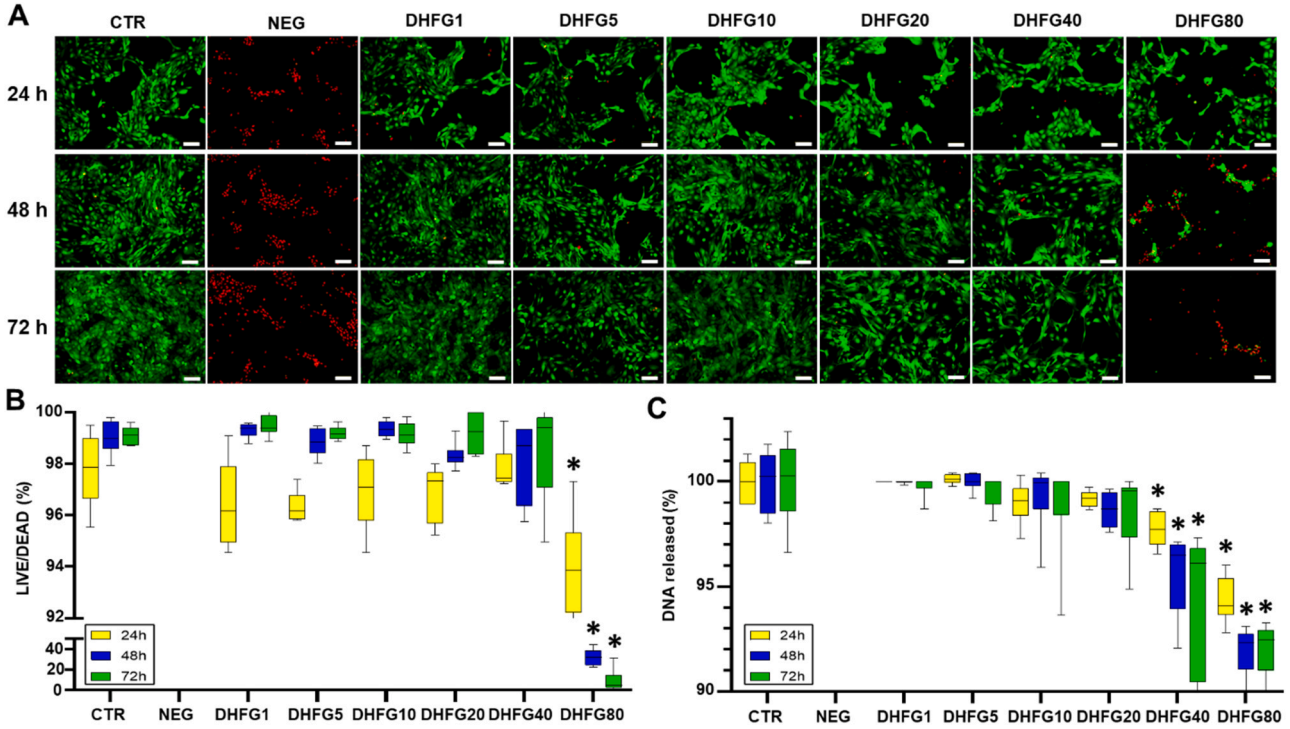

Fig. 2. Analysis of cell viability in human skin keratinocytes cultured with different concentrations of DL-3,4-Dihydroxyphenyl glycol (DHFG) (1, 5, 10, 20, 40 and 80 μg/mL) for 24, 48 and 72 h. Cells cultured in basal medium without DHFG were considered as CTR. As negative control of cell death (NEG), cells were treated with 2% triton X-100. **(A)** Fluorescence microscopy images of cells incubated with LIVE/DEAD system. Live cells are stained in green and dead cells are stained in red. Scale bar: 100 μm. **(B)** Box and Whisker plot representation of quantification of live and dead cells treated with LIVE/DEAD kit. **(C)** Box and Whisker plot of quantification of DNA released to the medium by dead cells. In **(C)**, values were normalized regarding CTR group. Asterisks (*) refer to values with statistically significant differences with respect to the CTR group.

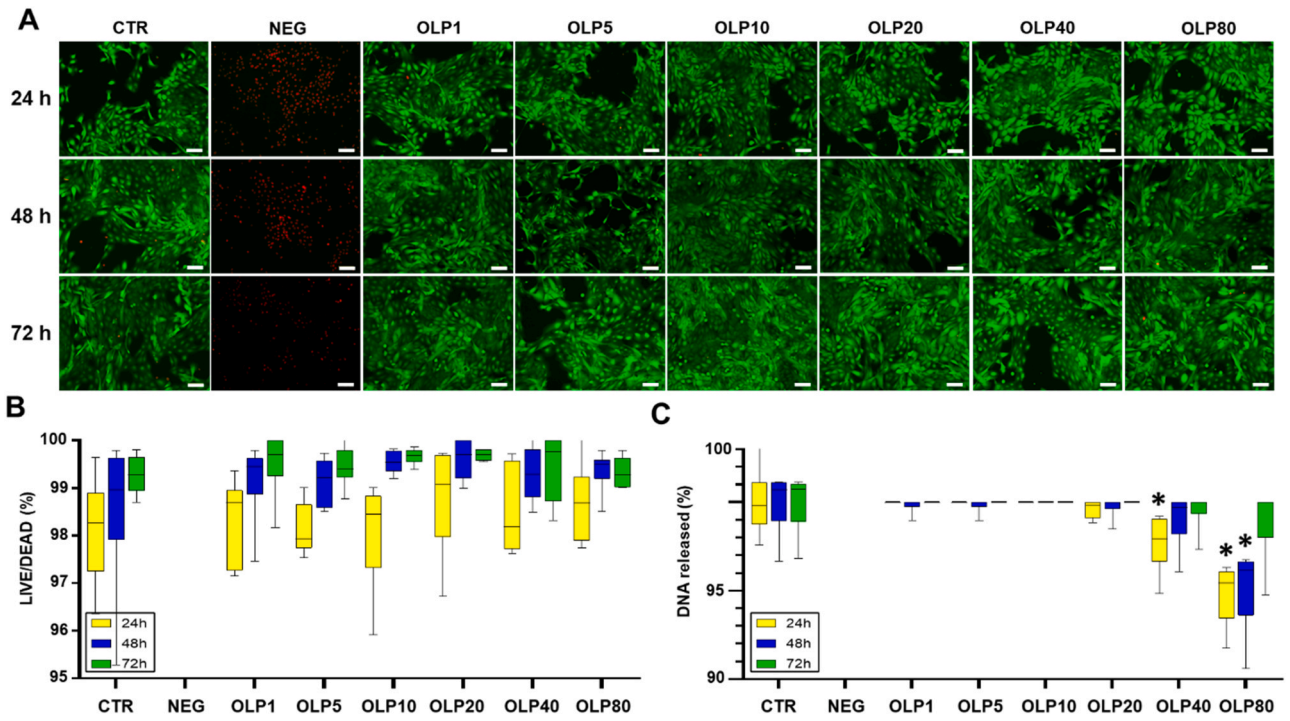

Fig. 3. Analysis of cell viability in human skin keratinocytes cultured with different concentrations of oleuropein (OLP) (1, 5, 10, 20, 40 and 80 μg/mL) for 24, 48 and 72 h. Cells cultured in basal medium without OLP were considered as CTR. As negative control of cell death (NEG), cells were treated with 2% triton X-100. **(A)** Fluorescence microscopy images of cells incubated with LIVE/DEAD system. Live cells are stained in green and dead cells are stained in red. Scale bar: 100 μm. **(B)** Box and Whisker plot representation of quantification of live and dead cells treated with LIVE/DEAD kit. **(C)** Box and Whisker plot of quantification of DNA released to the medium by dead cells. In **(C)**, values were normalized regarding CTR group. Asterisks (*) refer to values with statistically significant differences with respect to the CTR group.

significant association between DNA and the concentration of bioactive factors ($p < 0.0001$ for PE, DHFG, or OLP), but not with the culture time ($p = 0.2342$ for PE, $p = 0.9908$ DHFG, and $p = 0.2415$ for OLP), and interaction between both factors was not significant ($p = 0.9984$ for PE, $p = 0.9999$ for DHFG, and $p = 0.9771$ for OLP). Then, the ART-ANOVA results showed that global differences among all study groups were statistically significant for PE, DHFG, or OLP (Supplementary Tables S1-S4). Statistical comparisons between specific groups showed that all concentrations below 40 μg/mL were similar to CTR at all times. However, the concentration of 40 μg/mL and 80 μg/mL significantly reduced viability at 24 h for PE, DHFG and OLP, whereas the concentration of 80 μg/mL significantly reduced cell viability at 48 h for all compounds, and DHFG showed significantly lower cell viability values for DHFG40 and DHFG80 at all times (Supplementary Tables S1-S4).

In summary, these results suggest that most of the conditions evaluated in this work were associated to high cell viability, especially for the lowest concentrations (1–20 μg/mL).

3.2. *Analysis of cell proliferation of human keratinocytes cultured with PE, DHFG, or OLP*

We first quantified the number of cells obtained after each incubation using flow cytometry to determine the proliferative effects of PE, DHFG, or OLP on human keratinocytes (Supplementary Table S1). Analysis of the results using two-way ANOVA revealed statistically significant differences for the concentration of all three compounds ($p < 0.0001$ for PE, DHFG and OLP), and for the culture time only for DHFG ($p = 0.0219$) and OLP ($p < 0.0001$) but not for PE ($p = 0.2126$). Interaction between both parameters was non-significant for the three bioactive compounds analyzed here ($p = 0.9916$ for PE, $p = 0.9578$ for DHFG and $p = 0.4373$ for OLP). ART-ANOVA showed that the global differences among groups considered altogether were statistically significant ($p < 0.0001$ for all compounds and times). When specific groups of study were compared using Mann-Whitney statistical tests, we found some differences for specific groups. As shown in Fig. 4 A, a significant increase in the cell population was observed in the PE10 group at 48 h ($p = 0.0022$) and 72 h ($p = 0.0022$), whereas a significant decrease was observed in cells cultured with the highest concentrations of PE (40 and 80 μg/mL). Additionally, we found that DHFG (Fig. 4B) could significantly increase the number of cells only for DHFG1 at 48 h ($p = 0.0022$) and DHFG5 at 24 h ($p = 0.0411$). On the contrary, the highest concentrations of this compound reduced the number of cells in several study groups cultured with 20, 40, and 80 μg/mL of DHFG (Fig. 4C). Finally, our analysis of cells cultured with OLP (Fig. 4E) revealed a significant increase in the number of keratinocytes corresponding to the OLP5 group at 24, 48, and 72 h ($p = 0.0022$, $p = 0.0087$, $p = 0.0022$, respectively), and to the OLP10, OLP20, OLP40 and OLP80 groups at 72 h, although a significant reduction was found in OLP40 and OLP80 groups at 48 h (Supplementary Table S4). All study groups were significantly different to the NEG group, and some differences were detected for the comparison of specific groups (Supplementary Tables S1-S4).

In addition, we used the WST-1 reagent to determine cell proliferation (Supplementary Table S1), and we found several differences among groups. For the two-way ANOVA, results demonstrated the significant role of the concentration ($p < 0.0001$ for PE, DHFG and OLP), with the culture time being statistically significant only for OLP ($p < 0.0001$), but not for PE ($p = 0.1096$) or DHFG ($p = 0.7967$). Interaction was non-significant ($p = 0.9995$ for PE, $p = 0.8027$ for DHFG and $p = 0.9653$ for OLP). Global comparisons using ART-ANOVA was significant for all compounds and all culture times ($p < 0.0001$ for all conditions). When specific groups were compared, we found that PE used at a concentration of 1 and 10 μg/mL significantly increased WST-1 metabolic activity at 72 h ($p = 0.0411$ for both concentrations) (Fig. 4B), although the highest concentration of PE (80 μg/mL) significantly reduced this activity at all times. For DHFG (Fig. 4D), only DHFG20 at 72 h was

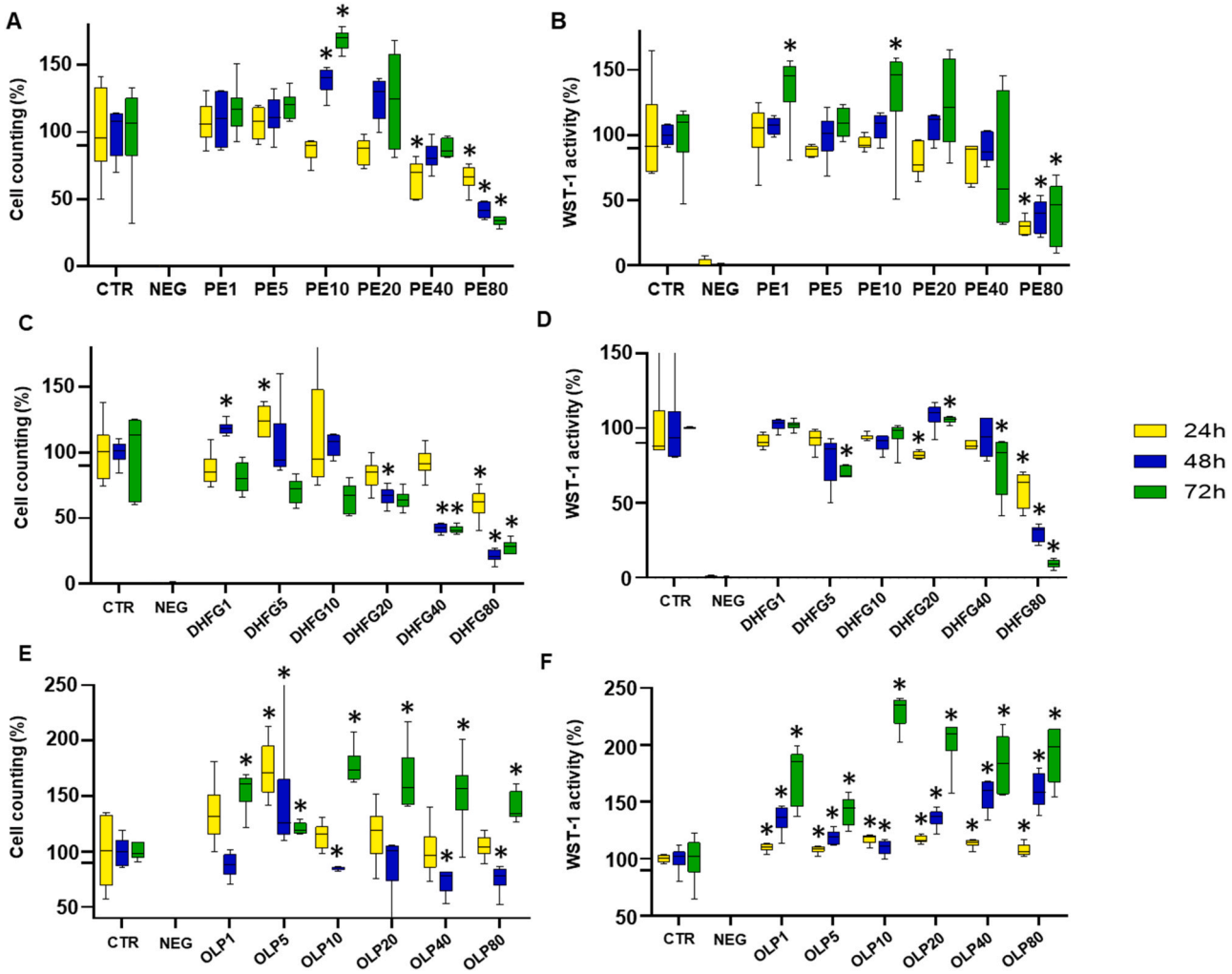

Fig. 4. Evaluation of cell proliferation on skin keratinocytes cultured with different concentrations (1, 5, 10, 20, 40 and 80 μg/mL) of phenolic extract (PE), DL-3,4-Dihydroxyphenyl glycol (DHFG) and oleuropein (OLP) for 24, 48 and 72 h, as determined by cell number quantification **(A, C, E)** and WST-1 metabolic activity **(B, D, F)**. In all cases, CTR and NEG groups were considered as 100% and 0%, respectively. Values were normalized with respect to CTR and represented as Box and Whisker plots. Statistically significant values compared to CTR are labeled with asterisks (*).

associated with a significant increase in WST-1 activity. However, several conditions significantly decreased metabolic activity and cell proliferation, especially, DHFG80 at all tested times. Finally, our analysis of WST-1 activity in cells cultured with OLP (Fig. 4F) showed that all concentrations of OLP significantly stimulated cell proliferation at 24, 48, and 72 h ($p < 0.05$ for all comparisons).

In general, these results suggest that specific concentrations and incubation times (especially, PE10 and OLP5) are able to increase cell proliferation as determined by cell counting and WST-1 analyses.

3.3. Analysis of viability and proliferation of cells cultured in a combination of PE and OLP treatments

Once we evaluated the effects of PE, DHFG, or OLP used separately, and after we determined the specific treatments and concentrations which were able to increase cell proliferation without any cytotoxic effects (PE10 and OLP5), we carried out an analysis of the cells cultured with a combination of the two conditions (PE10 and OLP5) to determine whether a combination of both products (PE-OLP) could synergistically improve the results. DHFG was not used in the subsequent analyses based on the results of the experiments described above. Results showed that PE-OLP had no effects on cell viability, and human keratinocytes cultured in this mixture were viable at 24, 48, and 72 h, with non-significant differences as compared to that of the CTR group ($p > 0.05$). Regarding cell proliferation, our results revealed that the number of cells found after each incubation time (24, 48, and 72 h) did not differ from that in the control group ($p > 0.05$) when flow cytometry was used. In addition, analyses of cell proliferation and metabolic activity using WST-1 showed no differences as compared with the CTR at 72 h and a significant reduction after 24 h and 48 h ($p < 0.0001$ and $p = 0.0003$, respectively). As shown in Fig. 5, the use of PE-OLP did not affect cell viability, but these combined compounds were not able to improve cell proliferation.

3.4. Analysis of the pro-proliferative effects of selected treatments on primary cultures of human native skin keratinocytes (HNSK)

To determine whether the conditions we selected in immortalized keratinocytes (PE10 or OLP5) could exert their pro-proliferative effects on HNSK, we established primary cultures of HNSK using cell culture media enriched with PE10 or OLP5. When we analyzed the number of keratinocyte colonies in the culture plates after each incubation time (Fig. 6A and B), we first found that CTR samples showed an average and standard deviation of 0.6 ± 0.63 colonies per well (median and quartile range of 1 [0,1]) at day 14, 1.2 ± 1.01 (1 [0.5–2]) colonies per well at day 21, and 1.47 ± 1.51 (1 [0–2.5]) colonies per well at day 28 of

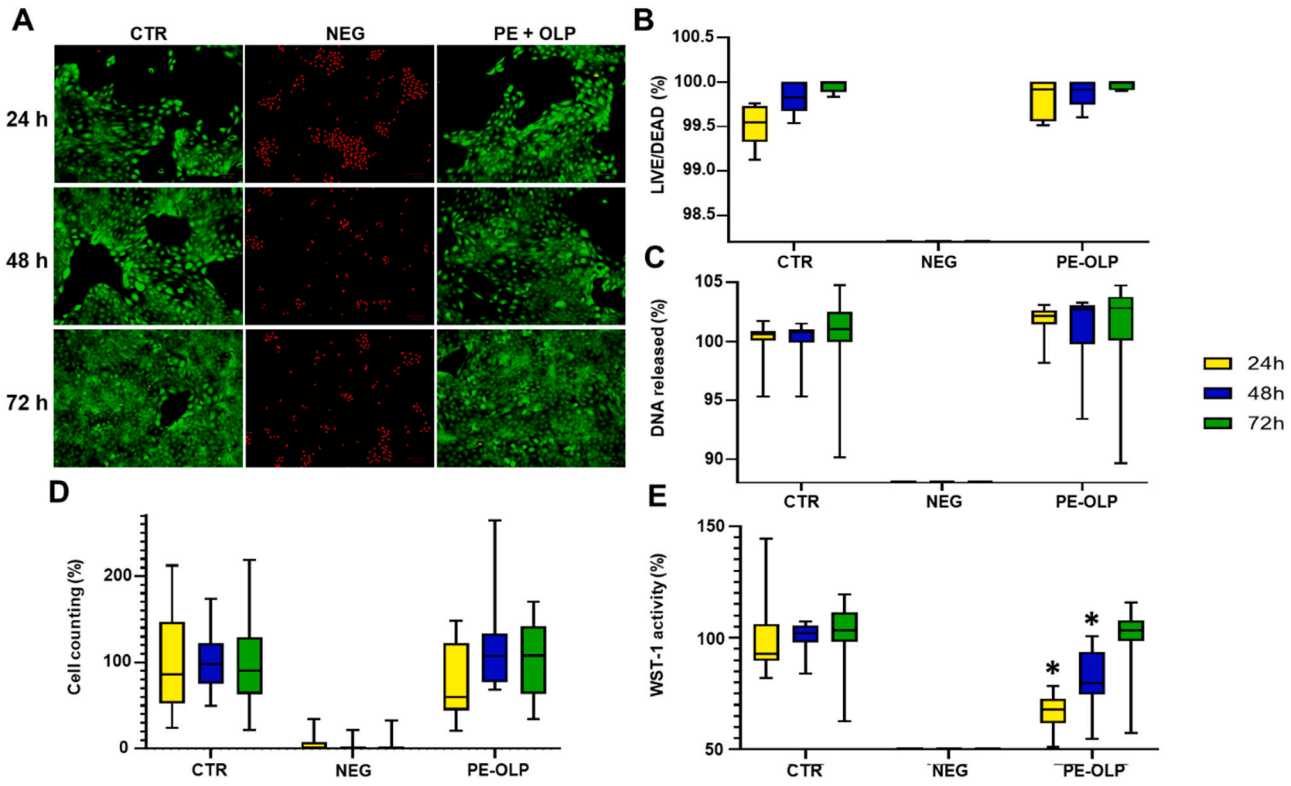

Fig. 5. Analysis of cell viability and proliferation of human skin keratinocytes cultured with the mixture of 10 μg/mL of phenolic extract (PE) and 5 μg/mL of oleuropein (OLP) (PE-OLP) for 24, 48 and 72 h. **(A)** Fluorescence microscopy images of cells stained with LIVE/DEAD reagents. Live cells are stained with green and dead cells are marked in red. Scale bar: 100 μm. **(B)** Quantification of live and dead cells exposed to LIVE/DEAD kit. **(C)** Quantification of DNA released to media by dead cells. **(D)** Quantification of cell number using flow cytometry. **(E)** Quantification of cell proliferation using WST-1 assay. In all cases, CTR and NEG groups were considered as 100% and 0%, respectively. All values were normalized with CTR and are represented as Box and Whisker plots. Statistically significant differences with CTR are marked with asterisks (*).

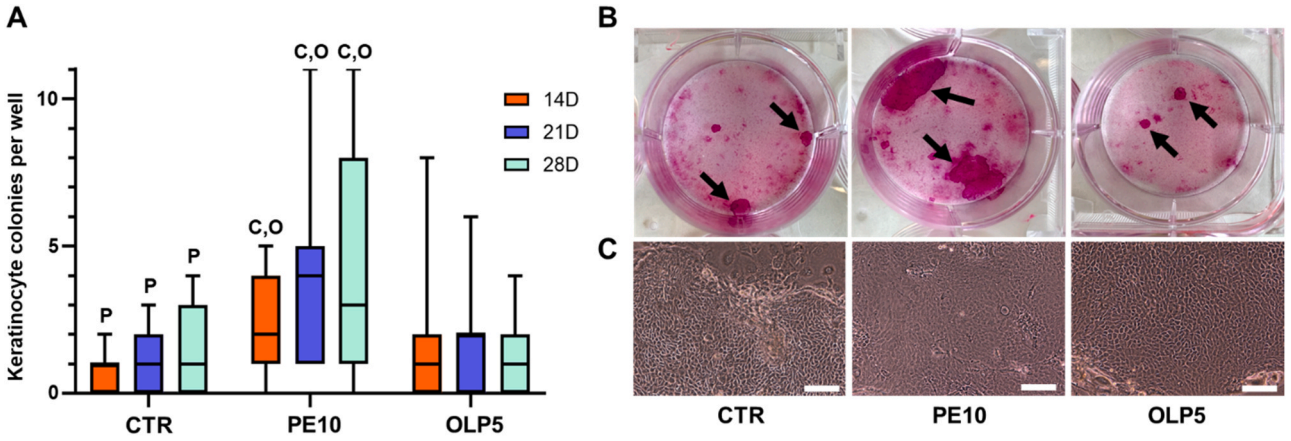

Fig. 6. Analysis of the effect of 10 μg/mL of phenolic extract (PE10) or 5 μg/mL of oleuropein (OLP5) on primary cultures of human native skin keratinocytes (HNSK) after 14, 21 and 28 days. Positive control (CTR) corresponds to cells incubated with medium devoid of any of these compounds. **(A)** Analysis of the number of keratinocyte colonies. Values labeled with "P" refer to statistically significant changes with respect to PE10, whereas differences with OLP5 are labeled with "O" and differences with CTR are labeled with "C". **(B)** Macroscopical images of normal keratinocytes colonies cultured for 28 days in each culture medium (CTR, PE10 or OLP5) and stained with hematoxylin-eosin. Different colonies are highlighted with arrows. **(C)** Phase-contrast microscopy images of illustrative keratinocyte colonies in each condition. Scale bar: 100 μm.

follow-up. Strikingly, we found that this number was significantly higher in cells cultured with PE10, which showed 2.4 ± 1.45 (2 [1–3.5]) colonies per well at day 14 ($p = 0.0004$ vs. CTR), 4.07 ± 3.37 (4 [1–5]) colonies per well at day 21 ($p = 0.0037$ vs. CTR), and 4.53 ± 3.78 (3 [1.5–8]) colonies per well at day 28 ($p = 0.0164$ vs. CTR). However, the use of OLP5 was not able to increase the number of cell colonies, and differences compared to that in the CTR group were not statistically significant at any of the three follow-up times ($p > 0.05$), with 1.27 ± 2.05 (1 [0–2]) colonies per well at day 14, 1.8 ± 1.78 (2 [0–2]) colonies per well at day 21, and 1.27 ± 1.16 (1 [0–2]) colonies per well at day 28. The two-ways ANOVA analysis revealed a significant association between the colony number and the culture condition (CTR, PE10 or OLP5) ($p < 0.0001$), but not with the culture time (0.1099), with the interaction being non-significant ($p = 0.9489$). For the pairwise

analysis, we found that PE10 was significantly higher than CTR at all study times ($p = 0.0004$ at 14 days, $p = 0.0037$ at 21 days and $p = 0.0164$ at 28 days of follow-up). However, differences between CTR and OLP5 were non-significant at all times ($p > 0.05$), whereas PE10 was significantly higher than OLP5 ($p = 0.0128$ at 14 days, $p = 0.0453$ at 21 days and $p = 0.0099$ at 28 days of follow-up).

Proliferation efficiency was also assessed by quantifying the area occupied by the grown keratinocytes at the end of the study. After 28 days of follow-up, we found that an average and standard deviation of $4.39 \pm 2.12\%$ (median and quartile range of 4.39 [3.64–5.14]) of the culture surface was occupied by keratinocytes in the CTR group, corresponding to 87.800 ± 42.400 cells, and $18.08 \pm 3.83\%$ (19.86 [16.78–20.29]) of the surface, corresponding to 361.600 ± 76.600 cells in the PE10 group and $4.61 \pm 4.25\%$ (4.61 [3.11–6.11]), corresponding to 92.200 ± 85.000 cells in the OLP5 group (Fig. 6B). Differences between PE10 and both the CTR and OLP5 groups were statistically significant ($p = 0.0026$ and $p = 0.0067$, respectively), but the area occupied by the keratinocytes in OLP5 group was similar to that in the

Table 1
Gene expression analysis of primary cultures of human native skin keratinocytes (HNSK) cultured for 28 days in control medium (CTR), PE10 or OLP5. For each gene, the fold-change expression is shown as average ± standard deviation and median and quartile range, along with the statistical p value and the relative regulation of the gene as compared to CTR (UP: upregulated, DO: downregulated, NC: no change).

Gene type	Gene symbol	Gene name	CTR vs. PE10 fold-change	CTR vs. PE10 regulation	CTR vs. OLP5 fold-change	CTR vs. OLP5 regulation
Proliferation	*MKI67*	Marker of proliferation KI67	1.78 ± 1.07 / 1.53 [1.08–1.78]	UP ($p = 0.0411$)	1.49 ± 0.52 / 1.49 [1.02–1.86]	NC ($p > 0.05$)
	PCNA	Proliferating cell nuclear antigen	1.69 ± 0.8 / 1.47 [1.24–1.67]	UP ($p = 0.0152$)	1.5 ± 0.37 / 1.57 [1.22–1.7]	UP ($p = 0.0260$)
Epithelial differentiation	*BGN*	Biglycan	1.91 ± 1.15 / 1.45 [1.3–1.88]	UP ($p = 0.0411$)	1 ± 0.33 / 0.95 [0.76–1.13]	NC ($p > 0.05$)
	FLG	Filaggrin	0.55 ± 0.3 / 0.58 [0.31–0.72]	DO ($p = 0.0411$)	4.99 ± 5.32 / 4.03 [1.13–6.12]	NC ($p > 0.05$)
	IVL	Involucrin	0.56 ± 0.39 / 0.52 [0.28–0.85]	NC ($p > 0.05$)	1.27 ± 0.91 / 1.08 [0.56–1.57]	NC ($p > 0.05$)
Basement membrane	*COL4A1*	Collagen type IV Alpha 1 chain	1.52 ± 1.12 / 1.01 [0.74–2.02]	NC ($p > 0.05$)	0.78 ± 0.13 / 0.79 [0.68–0.84]	NC ($p > 0.05$)
	LAMA1	Laminin Subunit Alpha 1	1.6 ± 1.19 / 1.22 [1.11–1.61]	NC ($p > 0.05$)	1.38 ± 0.39 / 1.32 [1.1–1.46]	NC ($p > 0.05$)
	LAMA3	Laminin Subunit Alpha 3	0.71 ± 0.53 / 0.65 [0.29–1.06]	NC ($p > 0.05$)	0.66 ± 0.43 / 0.64 [0.41–0.82]	NC ($p > 0.05$)
	LAMB1	Laminin Subunit Beta 1	1.65 ± 1.12 / 1.94 [0.92–2.48]	NC ($p > 0.05$)	1.38 ± 0.68 / 1.18 [1.11–1.51]	NC ($p > 0.05$)
	LAMC1	Laminin Subunit Gamma 1	1.97 ± 1.41 / 1.67 [1.03–2.54]	NC ($p > 0.05$)	1.24 ± 0.28 / 1.25 [0.99–1.45]	NC ($p > 0.05$)
Intercellular junctions	*DSP*	Desmoplakin	0.59 ± 0.36 / 0.69 [0.32–0.82]	NC ($p > 0.05$)	0.92 ± 0.54 / 0.94 [0.46–1.3]	NC ($p > 0.05$)
	FN1	Fibronectin 1	2.45 ± 1.97 / 1.82 [1.18–2.46]	NC ($p > 0.05$)	1.35 ± 0.32 / 1.27 [1.2–1.5]	NC ($p > 0.05$)
	GJA1	Gap Junction Protein Alpha 1	1.47 ± 0.94 / 1.17 [1.04–1.18]	NC ($p > 0.05$)	0.93 ± 0.09 / 0.91 [0.86–0.99]	NC ($p > 0.05$)
	GJA4	Gap Junction Protein Alpha 4	1.74 ± 1.02 / 1.31 [1.1–2.25]	NC ($p > 0.05$)	2.61 ± 0.89 / 2.37 [1.89–3.29]	UP ($p = 0.0022$)
	JUP	Junction Plakoglobin	0.6 ± 0.35 / 0.81 [0.33–0.83]	DO ($p = 0.0260$)	0.44 ± 0.34 / 0.42 [0.19–0.57]	DO ($p = 0.0260$)
	PKP1	Plakophilin 1	0.59 ± 0.38 / 0.62 [0.29–0.92]	DO ($p = 0.0411$)	0.67 ± 0.43 / 0.62 [0.33–0.89]	NC ($p > 0.05$)
	PPL	Periplakin	0.91 ± 0.62 / 0.69 [0.45–1.18]	NC ($p > 0.05$)	0.72 ± 0.41 / 0.64 [0.46–1.01]	NC ($p > 0.05$)
	TJP1	Tight Junction Protein 1	1.38 ± 1.27 / 0.96 [0.57–1.54]	NC ($p > 0.05$)	0.98 ± 0.25 / 1.01 [0.81–1.18]	NC ($p > 0.05$)
	TJP2	Tight Junction Protein 2	1.38 ± 0.99 / 1.04 [0.81–1.26]	NC ($p > 0.05$)	1.18 ± 0.11 / 1.2 [1.09–1.24]	NC ($p > 0.05$)
Cytokeratins	*KRT1*	Cytokeratin 1	0.37 ± 0.3 / 0.4 [0.11–0.64]	DO ($p = 0.0260$)	2.53 ± 2.14 / 2.37 [0.78–4.37]	NC ($p > 0.05$)
	KRT10	Cytokeratin 10	0.3 ± 0.38 / 0.19 [0.07–0.31]	DO ($p = 0.0152$)	1.3 ± 1.17 / 1.09 [0.38–1.96]	NC ($p > 0.05$)
	KRT13	Cytokeratin 13	0.55 ± 0.37 / 0.62 [0.28–0.78]	NC ($p > 0.05$)	0.74 ± 0.55 / 0.61 [0.35–0.91]	NC ($p > 0.05$)
	KRT18	Cytokeratin 18	0.65 ± 0.43 / 0.56 [0.35–0.9]	NC ($p > 0.05$)	0.53 ± 0.23 / 0.54 [0.39–0.66]	DO ($p = 0.0043$)
	KRT19	Cytokeratin 19	0.93 ± 1.06 / 0.39 [0.3–1.18]	NC ($p > 0.05$)	0.51 ± 0.24 / 0.5 [0.42–0.52]	DO ($p = 0.0152$)
	KRT3	Cytokeratin 3	0.43 ± 0.33 / 0.41 [0.15–0.67]	DO ($p = 0.0087$)	1.76 ± 2.51 / 0.78 [0.28–1.72]	NC ($p > 0.05$)
	KRT5	Cytokeratin 5	0.63 ± 0.68 / 0.41 [0.18–0.91]	NC ($p > 0.05$)	1.01 ± 0.65 / 0.83 [0.56–1.51]	NC ($p > 0.05$)
	KRT6A	Cytokeratin 6A	0.56 ± 0.33 / 0.56 [0.34–0.82]	NC ($p > 0.05$)	1.2 ± 0.86 / 1.13 [0.51–1.69]	NC ($p > 0.05$)
	KRT7	Cytokeratin 7	0.95 ± 0.4 / 1 [0.69–1.29]	NC ($p > 0.05$)	1.22 ± 0.62 / 1.14 [0.76–1.76]	NC ($p > 0.05$)
	KRT8	Cytokeratin 8	0.91 ± 0.47 / 0.92 [0.6–1.19]	NC ($p > 0.05$)	0.55 ± 0.23 / 0.56 [0.37–0.71]	DO ($p = 0.0260$)
	KRTCAP2	Keratin Associated Protein 2	1.43 ± 0.79 / 1.22 [0.93–2.06]	NC ($p > 0.05$)	1.51 ± 0.5 / 1.28 [1.24–1.68]	NC ($p > 0.05$)

CTR samples ($p > 0.05$).

In summary, the use of PE10 was significantly associated to an increase in colony number and area, whereas OLP5 was comparable to CTR.

3.5. Effects of the selected treatments on HNSK gene expression

To analyze the effects of PE10 and OLP5 on HNSK gene expression, qRT-PCR was conducted for 30 different genes at the end of the follow-up period of 28 days (Table 1). First, we found that *MKI67* and *PCNA*, two genes involved in cell proliferation, were significantly upregulated in the presence of PE10,

with more than 60% overexpression for both genes, whereas cells cultured with OLP5 showed 50% overexpression only for *PCNA*.

We found that human keratinocytes incubated with PE10 showed a significant upregulation of the biglycan gene (*BGN*), as compared to that in the control cells, whereas six genes involved in epithelial differentiation (filaggrin -*FLG*-), intercellular junctions (junction plakoglobin -*JUP*- and plakophilin 1 -*PKP1*-), and cytokeratins 1, 3, and 10 (*KRT1, KRT3*, and *KRT10*) were significantly downregulated upon incubation with PE10. For cells corresponding to the OLP5 group, we found a significant upregulation of a gene related to the development of intercellular junctions (gap junction protein alpha 4 -*GJA4*-) and a significant downregulation of four genes: one of them plays a role in intercellular junctions (*JUP*), while the other three genes were cytokeratins 8, 18, and 19 (*KRT8, KRT18*, and *KRT19*). These results show a significant variation of some specific genes in each experimental condition.

4. Discussion

Optimization of the currently available cell culture methods is a major requirement of research in keratinocyte biology. Several novel culture protocols have been published, with the final goal of increasing the number of cultured keratinocytes in a reduced time. Recent reports suggest that using conditioned culture media enriched with specific biomolecules could contribute to the increased proliferation of human keratinocytes. Several researchers have demonstrated the pro-proliferative potential of natural bioactive molecules isolated from olive oil, especially, maslinic acid, which can accelerate keratinocyte proliferation in culture [22,23]. In the present work, we evaluated PE, DHFG and OLP on human skin keratinocyte cultures.

The three olive oil-derived compounds were selected due to several reasons. First, PE, DHFG and OLP, along with maslinic acid, previously evaluated by our group, are among the most commonly used compounds derived from olive oil. Secondly, different studies demonstrated the positive effects of these molecules on skin healing and regeneration, protection from aging, reduction of oxidative stress, and decrease of histological alterations [21], suggesting a potential role on human skin keratinocyte proliferation [21]. Although other bioactive agents should also be evaluated in future studies, these three compounds, along with maslinic acid, are promising molecules to improve current tissue engineering cell culture protocols.

First, we analyzed PE, DHFG and OLP from olive oil to determine if the use of these compounds on cultured human keratinocytes could be associated to any side effects, since this is the first requirement of future medicinal products intended for clinical use, and a requirement of all national medicine agencies [26]. In this regard, we found that all molecules were associated to high cell viability under most experimental conditions, except the highest concentrations tested here (40 and 80 μg/mL), which resulted in a significant reduction of cell viability at all tested times. In general, viability was significantly affected by the increasing concentrations of each compound, although the effect of the culture time revealed to be less important than the concentration. These results confirm previous reports showing that high concentrations of different olive oil-derived molecules and PE exert pro-apoptotic effects [23,27,28].

After evaluating an immortalized cell line obtained from human foreskin keratinocytes, we determined the pro-proliferative effects of PE, DHFG and OLP on normal human foreskin keratinocyte cultures. In general, we found that the concentration was strongly associated with the cell proliferation, whereas the culture time was relevant only for some compounds analyzed using specific analysis methods. In general, our results suggest that some specific conditions succeeded in increasing cell proliferation *ex vivo*. When the effects of these compounds on immortalized human keratinocytes were evaluated, we found that PE and OLP could produce a positive pro-proliferative effect at low concentrations (10 μg/mL for PE and 5 μg/mL for OLP). In addition, an interesting finding was that the effect was concentration-dependent, and the highest concentrations analyzed were associated with the opposite effect, resulting in a significant reduction in cell proliferation.

Surprisingly, our analysis of the mixture of PE10 and OLP5 revealed a complete absence of biological effects on keratinocyte proliferation. Although these results are intriguing, we hypothesize that the mixture of specific molecules found in PE is optimal for inducing keratinocyte cell proliferation; however, the combination with OLP, even when OLP is partially similar to specific molecules found in PE, could alter the natural balance among components and modify the effect of both molecules.

In this milieu, previous studies demonstrated that specific molecules with a positive biological effect showed an antagonistic response when combined with a single therapy [29]. Therefore, future studies should investigate why the compounds used in the present study did not exert any positive effect when combined.

Furthermore, we evaluated the two specific conditions showing positive results in our analysis on normal human skin keratinocyte primary cultures, since our final goal was to use these compounds to favor cell proliferation of keratinocyte cultures established from patient biopsies. For this reason, we reproduced the method used to generate keratinocyte cell cultures for clinical use from samples of patients with severe burns and evaluated PE and OLP concentrations, as these molecules showed the most promising results in the initial analysis.

The results obtained for HNSK confirmed that the use of certain olive oil components could contribute to improving keratinocyte cell proliferation, although the results partially differed from those obtained with immortalized cells. Interestingly, PE10 increased both the average number of keratinocyte colonies at each follow-up and the area of the cultured colonies at the end of the experimental period. However, these results confirm the initial hypothesis that certain derivatives of olive oil may enhance the current technology related to normal keratinocyte culturing from patient biopsies and, therefore, may contribute to reducing the time required to generate abundant keratinocyte cell populations for skin tissue engineering purposes. PE has been extensively used both *ex vivo* and in vivo to improve cell proliferation and differentiation, mitochondrial function, and metabolism, and animals fed with this extract were able to grow and develop faster than control animals [30]. Although the mechanisms involved in the biological functions of PE are not fully understood and require further investigation, different reports suggest that the main effects of this extract could be related to its antioxidant potential [31] and the capability to modulate signal transduction pathways [32]. In this regard, several reports suggest that the mechanism of action of olive oil-derived compounds could be related to an activation of several cyclin-dependent kinases and AKT phosphorylation [22,33].

In line with previous studies, we performed gene expression analysis to shed light on the mechanisms associated with this phenomenon. In agreement with the highest number and area of keratinocyte colonies found in samples cultured with PE10, we found that cells cultured with PE showed a significant increase in *MKI67* and *PCNA*, two key genes whose expression is directly correlated with cell proliferation [34]. Another gene showing significant overexpression in normal cultured keratinocytes is *BGN*, which plays important roles not only in the synthesis of a well-structured extracellular matrix in connective tissues

[35], but also in keratinocyte cell differentiation [36]. Although its exact role is still unknown, biglycan has been found on the cell surface of differentiated keratinocytes, especially in the spinous stratum of the epidermis, and is considered an important mediator of keratinocyte function [36]. Future works should determine if upregulation of BGN is related to the positive cell proliferation effects of these compounds.

In contrast, our gene expression analysis of keratinocytes cultured in PE10 revealed a decrease of six genes related to epidermal function, including several genes involved in cell-cell adhesion, cytokeratins, and epidermal differentiation. Regarding the three cytokeratin genes that became downregulated after PE10 culturing (*KRT1*, *KRT10* and *KRT3*), it is well known that these keratins are typically expressed by terminally differentiated native epithelia and are considered as markers of keratinocytic differentiation [37]. Similarly, we found that two desmosome-related genes were downregulated in cells cultured with PE10. Expression of genes encoding for the different components of the human desmosomes is also highly dependent on cell differentiation, and different studies carried out by our research group demonstrated that these genes become progressively upregulated as epithelial cells reach higher levels of cell differentiation in bioengineered models of human tissues generated by tissue engineering, including the human cornea, oral mucosa, and skin [38,39]. In this regard, it has been previously demonstrated that cell proliferation and differentiation are antagonistic processes, and the balance between them determines the final fate of the tissue [40]. Although most genes related to cell differentiation remained unchanged, some specific intercellular junction and cytokeratin genes were downregulated, indicating the possibility that PE10 could partially induce cultured cells toward a proliferative state instead of differentiation. In addition, our results suggest that PE10 culturing was not able to induce the abnormal expression of genes associated with undesired side effects, such as the induction of hyperkeratosis, as in the case of cells cultured with other compounds able to increase proliferation, that are typically associated with significant overexpression of most cytokeratin genes [41].

Regarding OLP, our results suggest that the positive biological functions driven by this molecule on immortalized keratinocytes could not be fully reproduced in normal primary cell cultures of human skin keratinocytes. Although *PCNA* was overexpressed and some other genes modified their expression upon culture with OLP5, a proliferative effect was not observed in keratinocytes cultured with OLP5. One possible explanation could be related to the different intrinsic potentials of normal keratinocytes and immortal keratinocytes, which are probably more prone to respond *ex vivo* to the composition of the culture medium and the different growth factors contained in it. The specific composition of the culture medium is a crucial factor associated with the proliferation capability of several immortalized cell lines [42].

The present study had several limitations. On the one hand, our study using normal human keratinocytes was carried out on human foreskin cells. As it is well known that foreskin keratinocytes may not behave as keratinocytes isolated from other sources, future studies should be conducted to determine the reproducibility of our results in other cell types. In addition, our analyses of normal skin cells were performed on primary keratinocyte cultures. As it is well known that normal cells may show different phenotypes and functions upon sequential subculturing [43], future studies should determine the potential role of these compounds in advanced cell passages of HNSK. On the other hand, our study should be complemented with the analysis of protein levels, and future studies should be carried out to determine if cells cultured using these compounds retain their normal differentiation capability. Finally, the results of the present study should be confirmed in a real scenario using wider ranges of conditions and concentrations to determine whether these compounds can improve the protocols used for generating bioartificial human skin substitutes in patients with severe burns.

Conclusion

Our results suggest that using PE from olive oil at a concentration of 10 μg/mL could increase the proliferative potential of human keratinocytes without altering cell viability and functions. These results open the door to its future use to accelerate the generation of bioartificial substitutes for human skin. Furthermore, shortening the time required for the biofabrication process could offer earlier treatment for patients affected by severe skin burns and, therefore, increase patient survival.

Ethics statement

All patients or their legal representatives provided informed consent for participation in the study. The study was conducted following the Declaration of Helsinki and approved by the Institutional Research and Ethics Committee in Biomedical Research of Granada (Comité Ético de Investigación, CEIM/CEI) with protocol code S1900527, date of approval December 27, 2019, and 0711-N-20, date of approval April 28, 2020.

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CRediT authorship contribution statement

Conceptualization, MD.M-G, C.G-L, and M.A; study design and methodology, O.O-A and M.A; investigation, O.O-A; data analysis F.B-C and I.G.; writing-original draft, O.O-A and M.A; writing-review and editing, O.O-A, MD.M-G, C.G-L, I.G, and M.A. All authors have approved the final version of the manuscript.

Declaration of Competing Interest

Authors declare that they do not have any conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115000.

References

[1] A. Chaudhari, K. Vig, D. Baganizi, et al., Future prospects for scaffolding methods and biomaterials in skin tissue engineering: a review, IJMS 2016 (1974) 17.
[2] L.B. Naves, C. Dhand, L. Almeida, et al., *In vitro* skin models and tissue engineering protocols for skin graft applications, Essays Biochem. 60 (2016) 357–369.
[3] R.A. Kamel, J.F. Ong, E. Eriksson, et al., Tissue engineering of skin, J. Am. Coll. Surg. 217 (2013) 533–555.

[4] Y. Wang, J. Beekman, J. Hew, et al., Burn injury: challenges and advances in burn wound healing, infection, pain and scarring, Adv. Drug Deliv. Rev. 123 (2018) 3–17.
[5] T. Biedermann, S. Boettcher-Haberzeth, E. Reichmann, Tissue engineering of skin for wound coverage, Eur. J. Pedia Surg. 23 (2013) 375–382.
[6] D.K. Ozhathil, M.W. Tay, S.E. Wolf, et al., A narrative review of the history of skin grafting in burn care, Medicina 57 (2021) 380.
[7] M.A. Nilforoushzadeh, M.A. Amirkhani, E. Khodaverdi, et al., Tissue engineering in dermatology - from lab to market, Tissue Cell 74 (2022), 101717.
[8] J.J. Egea-Guerrero, G. Carmona, E. Correa, et al., Transplant of tissue-engineered artificial autologous human skin in andalusia: an example of coordination and institutional collaboration, Transplant. Proc. 51 (2019) 3047–3050.
[9] I. Garzón, M.A. Martin-Piedra, M. Alaminos, Human dental pulp stem cells. A promising epithelial-like cell source, Med. Hypotheses 84 (2015) 516–517.
[10] X. Zhang, W. Xu, X. Hu, Tissue-engineered skin regenerative units for epidermal keratinocytes expansion and wound healing, Med Sci. Monit. 27 (2021) 3, https://doi.org/10.12659/MSM.932978. Epub ahead of print 3 September 2021.
[11] M.A. Martin-Piedra, C.A. Alfonso-Rodriguez, A. Zapater, et al., Effective use of mesenchymal stem cells in human skin substitutes generated by tissue engineering, Eur. Cell Mater. 37 (2019) 233–249.
[12] A.L. Strong, M.W. Neumeister, B. Levi, Stem cells and tissue engineering, Clin. Plast. Surg. 44 (2017) 635–650.
[13] J. Wang, M. Mongan, X. Zhang, et al., Isolation and long-term expansion of murine epidermal stem-like cells, PLoS ONE 16 (2021), e0254731.
[14] Q. Chen, Y. Tang, H. Deng, et al., Curcumin improves keratinocyte proliferation, inflammation, and oxidative stress through mediating the SPAG5/FOXM1 axis in an in vitro model of actinic dermatitis by ultraviolet, Dis. Markers 2022 (2022) 1–23.
[15] S. Gunes, S. Tamburaci, M.C. Dalay, et al., *In vitro* evaluation of *Spirulina platensis* extract incorporated skin cream with its wound healing and antioxidant activities, Pharm. Biol. 55 (2017) 1824–1832.
[16] M. Kim, J. Kim, Y.-K. Shin, et al., Gentisic acid stimulates keratinocyte proliferation through ERK1/2 phosphorylation, Int J. Med. Sci. 17 (2020) 626–631.
[17] S. Miyata, Y. Oda, C. Matsuo, et al., Stimulatory effect of Brazilian propolis on hair growth through proliferation of keratinocytes in mice, J. Agric. Food Chem. 62 (2014) 11854–11861.
[18] R. Casas, R. Estruch, E. Sacanella, The protective effects of extra virgin olive oil on immune-mediated inflammatory responses, EMIDDT 18 (2017) 23–35.
[19] M. Gorzynik-Debicka, P. Przychodzen, F. Cappello, et al., Potential health benefits of olive oil and plant polyphenols, IJMS 19 (2018) 686.
[20] G. Marcelino, P.A. Hiane, K. Freitas, C. de, et al., Effects of olive oil and its minor components on cardiovascular diseases, inflammation, and gut microbiota, Nutrients 11 (2019) 1826.
[21] L. Melguizo-Rodríguez, A. González-Acedo, R. Illescas-Montes, et al., Biological effects of the olive tree and its derivatives on the skin, Food Funct. 13 (2022) 11410–11424.
[22] M. Abate, M. Citro, S. Pisanti, et al., Keratinocytes migration promotion, proliferation induction, and free radical injury prevention by 3-hydroxytirosol, IJMS 22 (2021) 2438.
[23] O. Ortiz-Arrabal, J. Chato-Astrain, P.V. Crespo, et al., Biological effects of maslinic acid on human epithelial cells used in tissue engineering, Front Bioeng. Biotechnol. 10 (2022), 876734.
[24] J. Chato-Astrain, D. Sánchez-Porras, Ó.D. García-García, et al., Improvement of cell culture methods for the successful generation of human keratinocyte primary cell cultures using EGF-loaded nanostructured lipid carriers, Biomedicines 9 (2021) 1634.
[25] C. Rasmussen, C. Thomas-Virnig, B.L. Allen-Hoffmann, Classical human epidermal keratinocyte cell culture, Methods Mol. Biol. 945 (2013) 161–175.
[26] L. Rico-Sánchez, I. Garzón, M. González-Andrades, et al., Successful development and clinical translation of a novel anterior lamellar artificial cornea, J. Tissue Eng. Regen. Med. 13 (2019) 2142–2154.
[27] M. Imran, M. Nadeem, S.A. Gilani, et al., Antitumor perspectives of oleuropein and its metabolite hydroxytyrosol: recent updates, J. Food Sci. 83 (2018) 1781–1791.
[28] N.S. Sani, H. Onsori, S. Akrami, et al., A comparison of the anti-cancer effects of free and PLGA-PAA encapsulated hydroxytyrosol on the HT-29 colorectal cancer cell line, Anticancer Agents Med. Chem. 22 (2022) 390–394.
[29] H. Ferjani, H. El Abassi, Ben, I. Salem, et al., The evaluate and compare the effects of the Tacrolimus and Sirolimus on the intestinal system using an intestinal cell culture model, Toxicol. Mech. Methods 26 (2016) 54–60.
[30] E. Gisbert, K.B. Andree, J.C. Quintela, et al., Olive oil bioactive compounds increase body weight, and improve gut health and integrity in gilthead sea bream (Sparus aurata), Br. J. Nutr. 117 (2017) 351–363.
[31] P. Kouka, G. Tsakiri, D. Tzortzi, et al., The polyphenolic composition of extracts derived from different Greek extra virgin olive oils is correlated with their antioxidant potency, Oxid. Med. Cell Longev. 2019 (2019) 1870965.
[32] D. Baci, M. Gallazzi, C. Cascini, et al., Downregulation of pro-inflammatory and pro-angiogenic pathways in prostate cancer cells by a polyphenol-rich extract from olive mill wastewater, Int J. Mol. Sci. 20 (2019) 307.
[33] B C de S Ribeiro, R V de C Faria, J de S Nogueira, et al., Olive oil promotes the survival and migration of dermal fibroblasts through Nrf2 pathway activation, Lipids 58 (2023) 59–68.
[34] X. Qiu, H. Wang, Z. Wang, et al., Expression of PCNA, Ki-67 and COX-2 in breast cancer based on DCE-MRI image information, J. Infect. Public Health 13 (2020) 2032–2037.
[35] S. Appunni, M. Rubens, V. Ramamoorthy, et al., Biglycan: an emerging small leucine-rich proteoglycan (SLRP) marker and its clinicopathological significance, Mol. Cell Biochem. 476 (2021) 3935–3950.
[36] P. Bianco, L.W. Fisher, M.F. Young, et al., Expression and localization of the two small proteoglycans biglycan and decorin in developing human skeletal and non-skeletal tissues, J. Histochem. Cytochem. 38 (1990) 1549–1563.
[37] H. Aldehlawi, S. Usman, A. Lalli, et al., Serum lipids, retinoic acid and phenol red differentially regulate expression of keratins K1, K10 and K2 in cultured keratinocytes, Sci. Rep. 10 (2020) 4829.
[38] I. Garzón, J. Miyake, M. González-Andrades, et al., Wharton's jelly stem cells: a novel cell source for oral mucosa and skin epithelia regeneration, Stem Cells Transl. Med. 2 (2013) 625–632.
[39] M. González-Andrades, I. Garzón, M.I. Gascón, et al., Sequential development of intercellular junctions in bioengineered human corneas, J. Tissue Eng. Regen. Med. 3 (2009) 442–449.
[40] L.J.A. Hardwick, F.R. Ali, R. Azzarelli, et al., Cell cycle regulation of proliferation versus differentiation in the central nervous system, Cell Tissue Res. 359 (2015) 187–200.
[41] C.A. Harrison, C.M. Layton, Z. Hau, et al., Transglutaminase inhibitors induce hyperproliferation and parakeratosis in tissue-engineered skin, Br. J. Dermatol. 156 (2007) 247–257.
[42] M. Combe, S. Sokolenko, Quantifying the impact of cell culture media on CHO cell growth and protein production, Biotechnol. Adv. 50 (2021), 107761.
[43] M.A. Martin-Piedra, I. Garzon, A.C. Oliveira, et al., Cell viability and proliferation capability of long-term human dental pulp stem cell cultures, Cytotherapy 16 (2014) 266–277.