ANTIMICROBIAL PROPERTIES OF OLIVE OIL PHENOLIC COMPOUNDS AND THEIR REGENERATIVE CAPACITY TOWARDS FIBROBLAST CELLS

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1. ABSTRACT

Some micronutrients of vegetable origin are considered potentially useful as wound-healing agents because they can increase fibroblast proliferation and differentiation.

The aim of this study was to evaluate the regenerative effects of selected olive oil phenolic compounds on cultured human fibroblasts and explore their antimicrobial properties.

Material and methods: The CCD-1064Sk fibroblast line was treated for 24 h with 10^{-6}M luteolin, apigenin, ferulic, coumaric acid or caffeic acid, evaluating the effects on cell proliferation by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) spectrophotometric assay; the migratory capacity by the scratch assay and determining the expression of Fibroblast Growth Factor (FGF), Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor- β1 (TGFβ1), Platelet Derived Growth Factor (PDGF), and Collagen Type I (COL-I) genes by real-time polymerase chain reaction. The antimicrobial capacity of the polyphenols was evaluated by the disc diffusion method.

Results: All compounds except for ferulic acid significantly stimulated the proliferative capacity of fibroblasts, increasing their migration and their expression of the aforementioned genes. With respect to their antimicrobial properties, treatment with the studied compounds inhibited the growth of *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Proteus spp.,* and *Candida Albicans*.

Conclusions: The phenolic compounds in olive oil have a biostimulatory effect on the regeneration capacity, differentiation, and migration of fibroblasts and exert major antibacterial activity. According to the present findings, these compounds may have a strong therapeutic effect on wound recovery.

KEY WORDS: olive oil phenolic compounds, fibroblasts, proliferation, antimicrobial and migratory capacity,

2. INTRODUCTION

Skin is the largest organ of the body (surface area of 1.5-2 m) and comprises three main layers: the epidermis, dermis, and hypodermis [1]. Damage to the skin activates mechanisms to close the wound and recover its functional status. Wound healing is a complex process characterized by inflammatory, proliferative, and remodeling phases [2]. Hemostasia at the start of the inflammatory phase involves fibrin clot formation and coagulation, for which platelets are largely responsible [3]. Platelets also release growth factors, including transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF), which are essential for the regeneration of damaged skin [2,4]. Leukocytes (neutrophils, monocytes, and macrophages) then migrate to the tissue, attracted by histamines, growth factors, and proinflammatory cytokines, among other molecules [5,6]. These leukocytes release chemotactic factors that promote the arrival of various cell populations to the damaged area [7,8]. These include dermal fibroblasts, which are derived from dermal progenitors [9] and are largely responsible for producing the elements that compose the extracellular matrix (e.g., collagen and fibronectin), contributing to the formation of granulation tissue [10]. These fibroblasts can sometimes differentiate towards myofibroblasts, whose main functions are related to wound contraction [11]. The proliferative phase is completed by angiogenesis and re-epithelialization processes [12,13]. During the remodeling phase, the matrix stabilizes and its constitutive elements are reorganized, with the degradation of fibroblast-secreted type III collagen and the increased production of type I collagen [14].

Wound healing can be affected by local factors such as oxygenation [15,16] or infections [17,18] and by systemic factors such as age [19,20], stress [21,22], diabetes [23,24], obesity [25,26], drug consumption [27–29], or nutrition [30–32]. Thus, various micronutrients have been found to influence wound healing, including vitamins A [33],

C [34], and E [35], whose antioxidant capacity has been associated with greater fibroblast proliferation and differentiation and increased collagen and hyaluronic acid production, and whose deficiency has been associated with reduced angiogenic activity and greater capillary fragility [36,37]. The phenolic compounds of olive oil have also demonstrated antioxidant capacity through their action as chain breakers, donating hydrogen radicals to alkylperoxyl radicals [38,39] produced by lipid oxygenation and giving rise to the formation of stable derivatives during the reaction. These properties have attracted the attention of the food and drug industry to the phenolic compounds in olive oil as possible nutraceuticals, with the potential to protect against chronic, degenerative, and oxidative stress-related diseases [40–42].

However, despite the myriad of potential health benefits of olive oil, there is a gap of data published on the possible effects of olive oil phenolic fraction on fibroblasts, especially over the last two year. Despite of *in vitro* studies limitations our results provide new knowledge about molecular mechanism of action of olive oil phenolic compounds on fibroblasts, which suggest that these compounds could be useful to enhance wound healing. Thus, the objective of this study was to evaluate the regenerative effects of selected olive oil phenolic compounds on cultured human fibroblasts and explore their antimicrobial properties.

3. METHODOLOGY

3.1 CHEMICAL PRODUCTS AND REAGENTS

Standards of apigenin, luteolin, caffeic acid, p-coumaric acid and ferulic acid were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions of phenolic compounds were prepared in ethanol and stored at -20 °C. All solvents were of analytical or HPLC

grade (Sigma-Aldrich) and water was of Milli-Q quality (Millipore Corp, Bedford, MA, USA).

3.2 CELL CULTURE

The CCD-1064Sk typified human fibroblast cell line was purchased from American Type Cultures Collection (ATCC, Manassas, VA). It was kept in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 50 µg/mL gentamicin (Braum Medical SA, Jaen, Spain), 2.5 µg/mL amphotericin B (Sigma, St Louis, MO, USA), 1% glutamine (Sigma, St Louis, MO, USA), 2% HEPES (Sigma), and supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK). Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were detached from the culture flask with a solution of 0.05% Trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma) and then washed and suspended in complete culture medium with 10% FBS.

3.3 CELL PROLIFERATION ASSAY

The MTT colorimetric method was used to determine proliferation (Sigma-Aldrich Chemie), measuring the chemical reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into formazan, which is directly proportional to the number of viable cells in the culture. Fibroblasts were seeded at 1 x 10^4 cells/mL per well in a 96-well plate (Falcom, Becton Dickinson Labware, New Jersey) on estrogen-free culture medium without FBS and were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. The medium was then replaced with DMEM containing phenolic compounds at 10^{-6} M. All experiments included cells incubated under the same

conditions without treatment compounds, as internal controls. Three separate experiments were performed for each treatment, and every experiment was performed at least in triplicate. On completion of the treatment, the medium was replaced with DMEM without phenol-red, containing 0.5 mg/mL MTT (Sigma), and was incubated for 4 h. Cellular reduction of the tetrazolium ring of MTT resulted in the formation of a dark-purple water-insoluble deposit of formazan crystals. After incubation, the medium was aspirated and DMSO was added to dissolve the crystals. Absorbance was measured at 570 nm with a spectrophotometer (Sunrise TM, Tecan, Männedorf, Switzerland). The percentage cell proliferation was calculated with respect to cell cultures treated with ethanol alone (controls) [43].

3.4 STUDY OF THE EFFECT OF DIFFERENT PHENOLIC COMPOUNDS ON THE GENE EXPRESSION OF FIBROBLASTS

Real-time polymerase chain reaction (RT-PCR) was used to determine the effect of the phenolic compounds under study on the gene expression of fibroblasts.

3.4.1 RNA Extraction and cDNA Synthesis (Reverse Transcription)

Cultured human fibroblasts were treated for 24 h with apigenin, luteolin, caffeic acid, p-coumaric acid or ferulic acid at 10^{-6} M. mRNA was extracted from the cells by a silicate gel technique using the Qiagen RNeasy extraction kit (Qiagen Inc., Hilden, Germany), which includes a DNAse digestion step. The amount of extracted mRNA was measured by UV spectrophotometry at 260 nm (Eppendorf AG, Hamburg, Germany), and contamination with proteins was determined according to the 260/280 ratio. An equal amount of RNA (1 μg total RNA in 40 μL total volume) was reverse-transcribed to cDNA

and amplified by PCR using the iScript™ cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA, USA), following the manufacturer's instructions [44].

3.4.2 Real-Time Polymerase Chain Reaction (RT-PCR)

The mRNA of FGF, VEGF, TGFβ1, PDGF, and COL-I was detected with primers designed using the NCBI-nucleotide library and Primer3-design (Table 1). All primers were matched to the mRNA sequences of the target genes (NCBI Blast software). The final results were normalized using ubiquitin C (UBC), peptidylprolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) as stable housekeeping genes [45].

Gene	Sense Primer	Antisense Primer	Amplicon (bp)
FGF	5´-CCCATATTCCCTGCACTTTG-3´	5´-ACCTTGACCTCTCAGCCTCA-3´	195
VEGF	5´-CCTTGCTGCTCTACCTCCAC-3´	5´-CACACAGGATGGCTTGAAGA-3´	197
TGF-β1	5´-TGAACCGGCCTTTCCTGCTTCTCATG-3´	5´-GCGGAAGTCAATGTACAGCTGCCGC-3´	152
COL-I	5'-CTGGCAAAGAAGGCGGCAAA-3'	5'-CTCACCACGATCACCACTCT-3'	503
PDGF	5´-CGGAGTCGGCATGAATCGCT-3´	5´-CTCCTTCAGTGCCGTCTTGT-3´	720

Table 1. Primer sequences for the amplification of cDNA by real-time PCR

3.5 STUDY OF THE EFFECT OF PHENOLIC COMPOUNDS ON THE MIGRATION OF CULTURED HUMAN FIBROBLASTS

The effect of phenolic compounds on fibroblast migration was analyzed by using the scratch assay in accordance with Moghadam et al (2019). Culture inserts were used, consisting of two wells separated by a wall, and 70 μL of cell suspension (10×10^4 cells/mL) was cultured in each well. After 24 h of attachment and full confluency, the culture inserts were removed to form a cell-free gap. Cells were washed with PBS to

remove cell debris and then supplemented with phenolic compounds at 10^{-6} M and incubated at 37 °C and 5% CO_2. Images were taken at 0, 4, 8 12, and 24 h post-treatment with a phase contrast inverted microscope. Motic Images Plus software (Motic, Hong Kong) was used to analyze the cell migration. The percentage wound closure was calculated by measuring the gap according to the formula 1, where Wn is the width of the gap after a given time interval and W0 is the initial width immediately after forming the scratch.

Formula 1. % wound closure = (W0−Wn)/W0×100

3.6 STUDY OF ANTIMICROBIAL CAPACITY

The antimicrobial capacity of phenolic compounds was evaluated by using the disc diffusion method. Strains of *Escherichia coli, Candida albicans, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus feacalis, Pseudomona aeuroginosa*, and *Proteus spp.* were cultured in Müeller Hinton Agar medium overnight at 37 ºC. A suspension in Tryptic Soy Broth (TSB, Becton Dickinson) was prepared from these cultures at a concentration of $1x10^{8}$ according to McFarland standards 0.5 (DO600 0.1). The suspension was subsequently spread using a sterile cotton swab on a plate with Müeller Hinton Agar medium and later dried under a laminar flow hood for 30 min. Sterile cellulose discs with a diameter of 5 mm, impregnated with 10 µL of 10^{-6} M apigenin, luteolin, ferulic acid, caffeic acid, or coumaric acid, were then placed on the agar surface, and the plates were incubated in a stove at 37 ºC for 24 h. After this time, the growth inhibition halo was measured with a ruler, using as control a disc impregnated with the maximum ethanol concentration utilized to dissolve polyphenols [46]. All assays were performed at least in triplicate.

3.7 STATISTICAL ANALYSIS

Mean values±standard deviations were calculated for all variables. Multiple t-tests were performed using Graph-Pad Prism 7.03 (La Jolla, CA, USA) to evaluate between-group differences, considering $p<0.05$ as significant. At least three experiments were performed in all assays and for each culture.

4. RESULTS

4.1 EFFECT OF OLIVE OIL PHENOLIC COMPOUNDS ON FIBROBLAST PROLIFERATION

Figure 1 depicts the proliferative effect on CCD-1064SK human epithelial fibroblast cell line at 24 h after treatment with the studied phenolic compounds, which significantly increased the proliferative capacity of fibroblasts ($p<0.0001$) *versus* controls by 38-81.2%, depending on the compound in question. Coumaric acid achieved the highest increase (81.2% vs. controls), followed by caffeic acid (77.1%), ferulic acid (65.0%), apigenin (55.8%), and luteolin (38.0%).

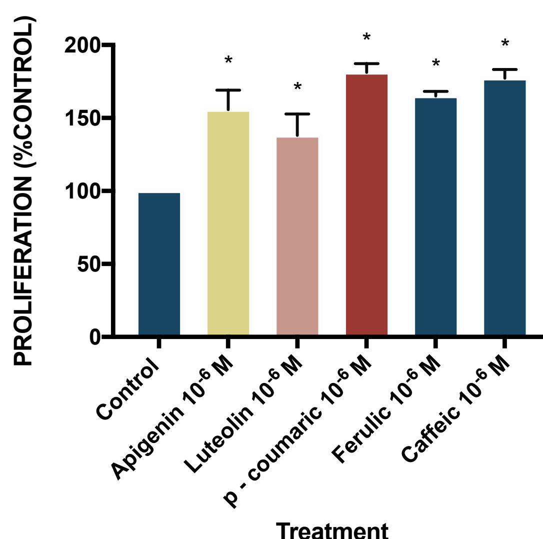

Figure 1. Effect of apigenin, luteolin, p-coumaric acid, ferulic acid and caffeic acid at a dose of 10-6 M on fibroblast proliferation after 24 h of incubation. Data are expressed as means + SD. *p ≤ 0.05

4.2 EFFECT OF PHENOLIC COMPOUNDS ON THE GENE EXPRESSION OF FIBROBLASTS

Figure 2 depicts the results of quantitative RT-PCR analysis of cells treated with 10^{-6} M luteolin, ferulic, coumaric, or caffeic acid for 24 h, showing a significantly increased expression of COL-I, PDGF, FGF, VEGF, and TGFβ1 (Fig. 2). Treatment with apigenin significantly increased the expression of COL-I, PDFG, and TGFβ1 *versus* controls but produced no change in the expression of FGF or VEGF.

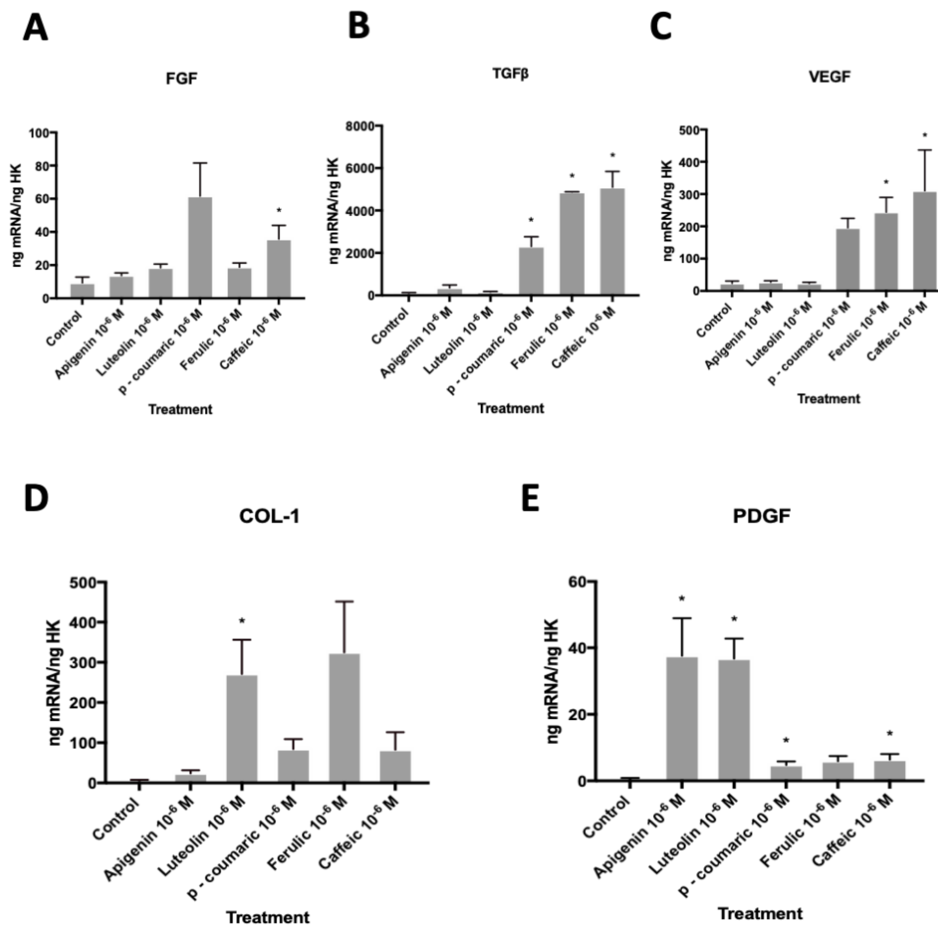

Figure 2. Expression of fibroblast genes (FGF, TGFβ1, VEGF, COL-I and PDGF) treated with apigenin, luteolin, p-coumaric acid, ferulic acid and caffeic acid at a dose of 10^{-6} M. Data are expressed as ng of mRNA per average ng of housekeeping mRNAs ± SD. $^{*}p \leq 0.05$

4.3 EFFECT OF PHENOLIC COMPOUNDS ON CELL MIGRATION

Figure 3 (A and B) depicts the scratch assay results on the effect of the phenolic compounds on fibroblast migration towards a cell-free space. Cells treated with 10^{-6} M apigenin, luteolin, caffeic, p-coumaric, or ferulic acid were analyzed at 4, 8, 12, and 24 h (Fig. 3.A).

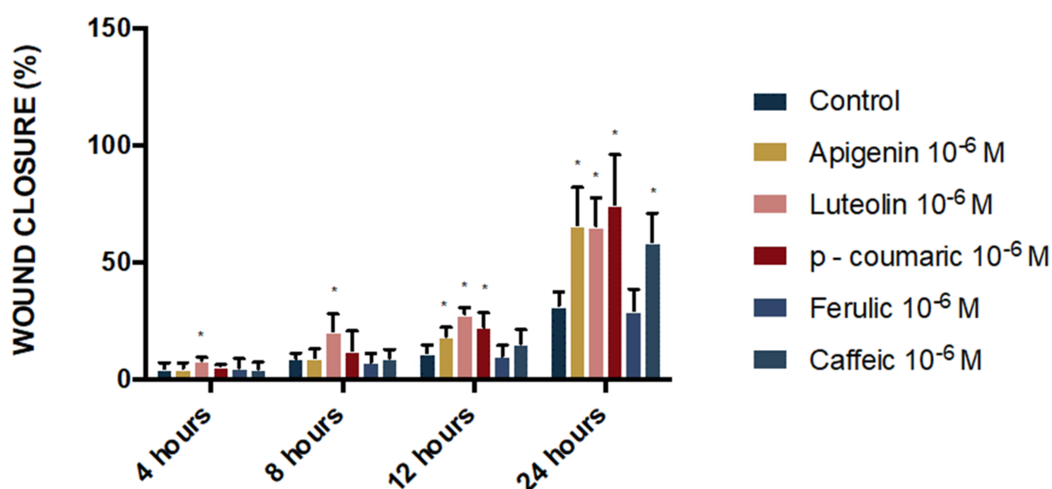

Figure 3A. Migration assay of phenolic compounds. Wound closure percentage of fibroblasts after different time intervals of exposure to phenolic compounds. Multiple t-tests were performed to determine the significance between each experimental group and control (* $p \leq 0.05$).

Luteolin alone significantly activated fibroblast migration from the beginning of treatment and at all time points (Fig. 3.B), being the only treatment to produce a significant increase in percentage closure *versus* controls at 4 h ($p<0.009$) and 8 h ($p<0.005$).

Luteolin, apigenin, and coumaric acid all significantly increased cell migration at 12 h of treatment, virtually doubling that of the control group. Finally, all studied phenolic compounds except ferulic acid showed a stimulating effect on fibroblast migration at 24 h of treatment, when the control treatment had reduced the scratch distance by 31.5%,

coumaric acid by 74.9%, apigenin by 66.2%, luteolin by 65.7%, and caffeic acid treatment by 58.8%.

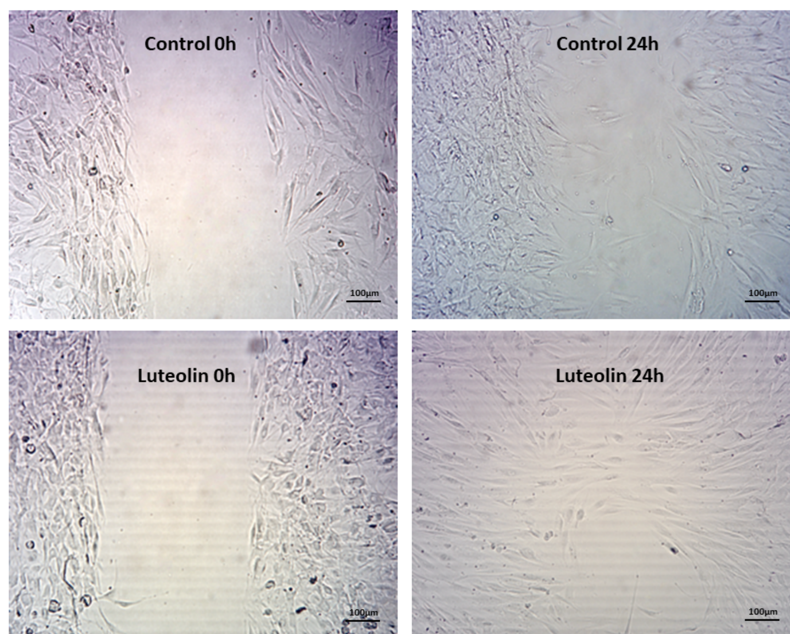

Figure 3B. Migration assay of phenolic compounds. Representative images of each treatment group after 24 h.

4.4 EFFECT OF PHENOLIC COMPOUNDS ON ANTIMICROBIAL CAPACITY

Treatment with the compounds under study produced growth halo inhibition against *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Proteus spp., and Candida albican,* and the largest inhibition halos were obtained by treatments with caffeic acid and coumaric acid (Table 2). Inhibition effects were especially marked against *Staphylococcus epidermidis, Proteus spp.,* and *Candida albicans.*

Table 2. Antimicrobial activities of apigenin, luteolin, p-coumaric acid, ferulic acid and caffeic acid at 10^{-6} M, against different microbial species (mm zone).

	Ferulic Acid		Cafeic Acid		Cumaric acid		Apigenin		Luteolin	
	Mean	**Sd**	**Mean**	**Sd**	**Mean**	**Sd**	**Mean**	**Sd**	**Mean**	**Sd**
Staphylococcus epidermidis	1,83	0,35	1,88	0,24	1,77	0,51	1,68	0,2	1,78	0,25
Staphylococcus aureus	0,9	0,2	1,02	0,16	1,12	0,30	1,02	0,13	0,95	0,27
Eschericia coli	0,87	0,06	0,83	0,06	0,87	0,15	0,90	0	0,93	0,06
Candida albicans	1,45	0,07	1,55	0,21	1,45	0,07	1,4	0,15	1,55	0,05
Proteus sp	1,13	0,06	1,23	0,06	1,30	0,1	1,23	0,06	1,23	0,15
Enterococcus feacalis	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Pseudomona Aeuroginosa	0,96	0,05	1	0	0,97	0,05	0,97	0,05	0,93	0,2

5. DISCUSSION

Wound regeneration can be enhanced by cell biostimulation or bacterial load control, among other approaches [47]. The results of this study demonstrate the regenerative potential of extra virgin olive oil phenolic compounds and their promotion of wound healing through the biostimulation of fibroblasts, augmenting their proliferative capacity and migration, increasing their expression of VEGF, PDGF, IGF, TGFβ1, and Col-I, and exerting antimicrobial activity to reduce the bacterial load.

Specifically, the proliferative capacity of cultured human fibroblasts was significantly increased by treatment with 10^{-6} M luteolin, apigenin, ferulic acid, coumaric acid, or caffeic acid for 24 h. In the same way, Hahn HJ et al (2016) found that treatment with ferulic acid protected human dermal fibroblasts from UVA-induced cell damage, favoring their proliferation without altering their cell cycle; the authors also associated

this treatment with an antioxidant effect through a reduction in dichlorofluorescein intensity and an alteration in the mRNA expression of superoxide dismutase 1 and catalase. They indicated that ferulic acid may act against the effects of aging on skin by modifying its physiological structure [48]. In another study, the viability of cultured human fibroblasts damaged by UVA-induced cytotoxicity was enhanced by treatment with 10 or 20 μM apigenin [49]. Among studies of phenolic compounds in other vegetable species, Sharma et al. demonstrated that the combination of curcumin and hyaluronic acid increased keratinocyte proliferation, reduced H_2O_2-induced oxidative damage, and improved cell migration in scratch wounds [50]. Compounds present in strawberries and blackberries have also been reported to reduce oxidative damage and increase the migration of fibroblasts by around 50% with respect to controls [51].

Treatment of osteoblasts with the same compounds has been found to increase their proliferative capacity and their expression of genes involved in cell differentiation and to modulate their surface antigens, with 10^{-6} M being the most active dose [41,52,53].

In the present study, *in vitro* treatment with phenolic compounds in olive oil significantly increased the expression of human fibroblast markers, including FGF, VEGF, TGFβ1, PDGF, and COL-I. The proteins encoded by these genes play an important role in wound healing by stimulating the proliferation, migration, and/or maturation of cell populations involved in the healing process, including fibroblasts, endothelial cells, and keratinocytes (Barrientos et al., 2014; Eckes et al., 2010; Gurtner et al., Sarvajnamurthy et al., 2013).

Wound repair is characterized by the formation of fibrovascular tissue, which contains fibroblasts, collagen, and blood vessels. The vascular component depends on angiogenesis, in which TGFβ and VEGF play important roles [58]. VEGF is considered to be the main factor involved in angiogenesis during wound healing, alongside collagen

production and epithelialization, while TGFβ is more important for polypeptide production and cell proliferation and differentiation in comparison to other growth factors [59]. In the present study, TGFβ expression was significantly increased by treatment with ferulic acid, caffeic acid, and coumaric acid. Platelet activation by prothrombin in response to skin damage would favor the release of EGF, IGF-1, PDGF, FGF, and TGFβ [60], which serve as biological signals to attract neutrophils, monocytes, leukocytes, and macrophages to mediate in the inflammation, protect the skin from infection, and secrete more growth factors to accelerate wound healing [7]. Frykberg et al. (2010) treated 65 refractory ulcers with growth factor concentrate and reported a reduction in ulcer area and volume in almost all cases after a mean of 2.8 weeks [61]. The above data confirm that the biostimulatory effects of phenolic compounds of extra virgin olive oil on fibroblasts, either alone or in combination with other products, can contribute to the regeneration of damaged tissue [62,63].

Collagen formation, an essential process in wound healing and contraction, is regulated by dermal and epidermal cells. In the present study, the studied compounds increased COL-I expression and accelerated fibroblast migration during the first hours of treatment, as demonstrated by the *in vitro* scratch assay (around double the closure rate vs. controls). The compounds favored increases in cell migration and in the expression of growth factor genes, inducing differentiation from fibroblasts to myofibroblasts, which participate in wound contraction [64]. In previous studies, the application of growth factor-rich plasma promoted the regeneration of damaged tissue [65], both alone and in combination with other products such as hyaluronic acid, achieving promising outcomes in the treatment of pressure ulcers [64].

Wound healing can be delayed by bacterial colonization, due both to leukocyte chemotaxis, with the consequent inflammatory response, and to interruption of the

migration and proliferation of fibroblasts and endothelial cells, among other relevant cell populations. Among all the microorganisms that can colonize a skin wound, the ones that are most frequently isolated are those analyzed in this study, such as *Escherichia coli, Candida albicans, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus feacalis, Pseudomona aeuroginosa,* and *Proteus spp* [66,67]. An outstanding feature in this type of wound is the formation of biofilms (polymicrobial biofilms) that are aggregates of microcolonies enclosed in an extracellular polymeric substance (EPS) that are distributed on the wound, intricate with fibroblasts or keratinocytes and the matrix extracellular (ECM), which communicate with each other through quorum sensing circuits (Quorum Sensing). Microbial cells embedded in their EPS develop in an optimal environment so that they evade the host's immune response and antibiotic action [67]. Infections have a negative impact on the wound healing process and on the health of patients, and may even be life-threatening [68–70]. All phenolic compounds under study demonstrated antimicrobial activity. Polyphenols from other sources have also shown effects against wound infections, including resveratrol, curcumin, chitosan, and the compounds present in pomegranates [71–74]. In this sense, recent researches have used multifunctional polyelectrolyte wound dressing membrane on the basis of chitosan, hyaluronan, phosphatidylcholine dihydroquercetin, for acute and chronic wounds treatment, showing regenerative, antioxidant, antimicrobial and anti-inflammatory properties, suggesting their utility in the treatment of wound healing [74–76]. Similarly, Omer et al. have tested the use of these compounds as nanocarriers for drugs that promote their effective encapsulation and subsequent slow release, in places that require their regenerative properties [77].

In conclusion, treatment with phenolic compounds in olive oil have a biostimulatory effect on cultured human fibroblasts, improving their regenerative

capacity, differentiation, and migration and exerting an important antibacterial action. According to the present findings, these compounds have high therapeutic potential for wound recovery; however, further *in vivo* studies in animals and humans are required to verify their clinical usefulness.

6. FUNDING SOURCES

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