Hydrolate *of Helichrysum italicum* Promotes Tissue Regeneration During Wound Healing

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Summary

Wound healing is a dynamic process involving different cell types with distinct roles according to the stages of healing. Fibroblasts and stem cells actively participate in tissue regeneration. A proper stimulation could contribute to enhance wound healing processes. *Helichrysum italicum (H. italicum*) is a medical plant well described for its pharmacological, antimicrobial, and anti-inflammatory activities. Aim of the present work was to examine the effect of the hydrolate derivate from *H. italicum* on stem cells isolated from skin and fibroblasts *in vitro* in presence or absence of tissue damage. The viability and proliferation of all cell types cultured in different conditions were analyzed by MTT and BrdU assays. Cell proliferation after wound was analyzed with scratch test. Also, the expression of the main genes involved in tissue repair was evaluated by RT-qPCR analysis. Here we describe the capability of hydrolate of *H. italicum* to promote tissue regeneration after scratch test both in stem cells and in fibroblasts. Moreover, the gene expression analysis revealed that, hydrolate of *H. italicum* is also able to enhance stemness related. In conclusion our results are encouraging, highlighting novel regenerative properties of hydrolate of *H. italicum* and paving the way for future application of this wasting product in accelerating wound healing.

Keywords

Stem cells • Fibroblasts • Tissue regeneration • Wound healing • molecular mechanisms • *Helichrysum italicum* • Phytochemistry • Skin • Plants • Bioactive molecules

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Introduction

Wound healing is a dynamic process in the human body resulting in a specific sequence of regulated events. It depends on many mediators and involves the spatial and temporal synchronization of different cell types with distinct roles according to the stages of healing: hemostasis, inflammation, proliferation, and tissue remodeling [1,2,3]. The main goal in wound healing is to heal the wound as quickly as possible [4]. The processes follow each other temporally but may also overlap [5], and all cellular events must be closely coordinated to effectively repair damaged tissue [6]. Several factors can influence the wound healing process: gender, age, hormones, stress, diabetes, obesity, infections, medications, alcoholism, smoking, and diet [7,8]. From the late inflammatory phase to final epithelialization, fibroblasts play a crucial role by secreting growth factors,

cytokines, collagens, and other extracellular matrix (ECM) components [9,10]. At the same time migration and proliferation of fibroblasts define the proliferative phase of repair in the healing process [11,12]. Also stem cells play a crucial role, being located in the "niche" and giving rise to cells responsible for specific functions in the healing process. Most stem cells have an epithelial origin and numerous mesenchymal stem cells can be found in the epidermis of undamaged skin, increasing during inflammation or tissue repair processes [13,14]. The skin comprises several types of stem cells including the skin stem cells (SSCs) used for this study, that can proliferate and replace damaged elements. Pluripotency of stem cells depends on expression of the so called “stemness genes”, in particular, SOX2, Oct-4 and NANOG, crucial transcription factors essential for the maintenance of the undifferentiated state [15,16]. Aged stem cells show a significant decrease in the expression of these stemness related genes, along with the loss of their differentiation and regenerative capabilities [17]. In adulthood both tissue regeneration and healing become increasingly difficult as stem cell activity is affected by cellular senescence, and stem cell niches may be compromised [16]. Within this context, the early regenerative events are related to tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine that mediates key roles in proliferation, differentiation, apoptosis, immune regulation, and induction of inflammation [18]. Moreover, the significant increase in the expression of caspase 8 (CASP8), involved also in wound healing processes, and hyaluronan synthase 2 gene (HAS2), related to the increased production of hyaluronic acid present in the extracellular matrix, appear to be crucial during tissue healing. The use of natural products, especially those derived from plants, is increasing in regenerative medicine because of their wide availability and fewer side effects [19,20]. Plant-derived extracts and their phytoconstituents, have been successfully described for their important effects on wound repair [21]. Their properties promote cell proliferation, increasing collagen synthesis, stimulating dermal reconstruction, and repairing the skin's lipid barrier function [22]. Among promising medical plants with pharmacological activity, *H. italicum* is already known for its antimicrobial and anti-inflammatory properties [23]. Plant species in the genus *Helichrysum (family Asteraceae)* are rich in bioactive secondary metabolites as, phenolic compounds, oligomers, and flavonoid glycosides, with different biological activities [1]. Aim of the present study was analyze the regenerative properties of hydrolate of *H. italicum* in accelerating wound healing and promoting tissue repair.

Materials and Methods

Preparation of hydrolate

Hydrolate of *H. italicum* (HH) was obtained by steam distillation of the flowering tops of *Helichrysum italicum* subsp. microphyllum (Willd.) harvested at the beginning of June 2021 from a crop grown on the 'LaNora Officinali' farm, located in the Municipality of Solarussa (Province of Oristano, Sardinia). The essential oil, separated from the hydrolate, contained a high amount of Curcumene (28 %), Neryl acetate (20 %) and Gamma-Muurolene (12 %), based on GC-MS analysis carried out one month after distillation.

Chemicals

Acetonitrile was purchased from Sigma-Aldrich Italy (IT) and were of the HPLC grade. Analytical standards of chlorogenic acid and naringenin were purchased from Extrasynthese (Lyon, France). Orthophosphoric acid (ACS ISO, for analysis, 85 %) were purchased from Carlo Erba. Water was distilled and filtered through a Milli-Q apparatus (Millipore, Milan, Italy) before use.

HPLC analysis

Qualitative and quantitative analyses of phenolic compounds in hydrolate were carried out using a method previously reported by Sarais *et al.* [24]. The equipment of analysis consisted of an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) with a quaternary pump, degasser system, autosampler integrated with a diode array detector (DAD) UV6000LP (Thermo Finnigan, Milan, Italy). Data were analyzed using ChromQuest version 4.0 software. The chromatographic separation was achieved with a Kinetex C18, 100 A (150 x 4.6mm, 5 μm particle size, Phenomenex) thermostated at 22 °C. Chromatographic separation of active ingredients was obtained using a gradient elution of mobile phase composed by an aqueous solution of phosphoric acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.4mL min -1. An increasing linear gradient was used starting from 5 % and reaching 80 % in 120 min of solvent B. The contents of the active ingredients were expressed as milligrams of active ingredient per L of extract. Data were expressed as the mean of three determinations ± standard deviation (SD).

Cell isolation and culturing

Human skin stem cells (SSCs) were obtained from biopsies of adult male and female patients after ethics committee approval (Ethical Clearance N. 0021565/2018, 22/03/2018-Commissione Etica CNR). They were isolated and cultured as previously described [16]. Human skin fibroblast 1 (HFF1) were purchased from ATCC (Manassas, VA, USA) and cultured in a Dulbecco's modified Eagle's Medium (DMEM) low-glucose medium (Life Technologies, Carlsbad, CA, USA), supplemented with 10 % fetal bovine serum (FBS Life Technologies, CA, USA), 2 mM l-glutamine (Euroclone, Milano, Italy) and 1 % of penicillin/streptomycin (Euroclone, Milano, Italy).

Experimental conditions

Fibroblasts and skin stem cells were divided in four different groups: the first two groups were cultured for 24 or 48 hours in the presence or absence of *HH* at different concentrations (40 %-5 % and 30 %-7,5 %). The other two groups were represented by fibroblasts or stem cells which after scratch test, undergo wound healing, in the presence or absence of *HH* at different concentration (40 %-10 %) (Fig. 1).

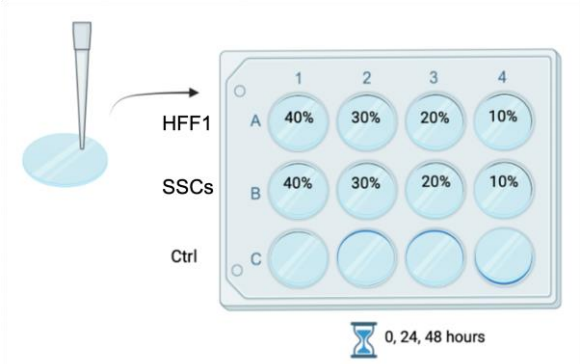

Fig. 1. Graphic representation of scratch test. HFF1 and SSCs were cultured for 24, and 48 hours in the presence of different concentrations of HH obtained with scalar dilutions. Control sample of each cell type was represented by untreated cells (Ctrl).

Evaluation of cell viability

The Thiazolyl Blue Tetrazolium Bromide assay (MTT) (Sigma-Aldrich, Saint Louis, MO, USA) was performed to evaluate the metabolic activity of HFF1 and SSCs. Cells were seeded at a concentration of 10000 cells/well in 96well plate and treated with scalar dilution of HH (40 %-5 % and 30 %-7.5 %) for 24 and 48h. After culturing in the conditions described above, MTT assay (Sigma-Aldrich) was performed. Cell viability was detected by a plate reader (OD570) and expressed as percentage of cell viability compared with untreated cells (control 100 %) and expressed as mean ± SD.

Evaluation of cytotoxicity and cell proliferation

The BrdU assay (#6813, Cell Signaling Technology, Euroclone, Milan Italy) is an immunoassay for the quantification of cell proliferation. To verify the potential of the HH in enhancing cell proliferation, the BrdU assay was performed with or without scratch test, as described above. Cells were seeded at a concentration of 6000 cells/well in 96-well plates and treated for 24h with the HH at the same concentration described above. During the experiment cells were not confluent. Cells cultured in the absence of HH samples were used as control. Cell viability was detected by plate reader (OD 450 nm) and were expressed in OD units compared with untreated cells (control 100 %). Data were expressed as mean ± SD, referring to the control.

Scratch assay

HFF1 and SSCs were seeded at a concentration of 45.000 cells/well in 12 well-plates and allowed to incubate until confluence. When cells were confluent, media was removed, and scratch was made in each well using a 200µl pipette tip (Fig. 1). Cells were washed in PBS to remove detached cells before adding the medium conditioned with different HH concentrations (40 %-10 %). Five different areas along the scratches of each well were analyzed by optical microscopy after 0, 24, 48 hours following the induced damage. The distance between each edge of the scratch was measured using the software ImageJ and expressed as percentage of closure of the area as compared to control untreated cells.

Real time-qPCR

Gene expression levels were detected by Real Time-qPCR. HFF1 and SSCs were exposed to the different concentration of HH after scratch test. Total mRNA was isolated using RNeasy Mini Kit (Qiagen, 40724 Hilden, Germany) according to the manufacturer's protocol. The quantity and purity of RNA were measured by OD 260/280 nm using a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). Then, 2.5 ng of RNA from each sample in triplicate was reverse-transcribed and amplified by a Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, 240 County Road Ipswich, MA, USA) via the Thermal Cycler (Bio-Rad, Hercules, CA, USA). The RT-qPCR analysis was performed for the following genes: stemness markers Oct-4, SOX2 and NANOG for SSCs, and TNF-α, HAS2, CASP8 genes for HFF1. All the

primers used were previously described [25] [26] [27]. The target Ct values were normalized HPRT1, considered as a reference gene, and mRNA levels were expressed as fold of change ($2^{-\Delta\Delta Ct}$) relative to the mRNA levels observed in untreated controls.

Statistical analysis

The experiments were performed two times with three technical replicates for each treatment. All statistical analyses were performed using unpaired Student's t-test, and, using SigmaStat v 3.5 software. Two-way analysis-of-variance ANOVA tests with Tukey's correction and the Wilcoxon signed-rank test were used. A $p \leq 0.05$ was considered statistically significant.

Results

Hydrolate of H. italicum is reach in phytochemicals

As previously reported, the characterization of the phenolic compounds was performed by HPLC-DAD analysis. A screening approach by selecting multiple wavelengths in a diode array detector allowed us to determine the presence of some hydrophilic phenols. Compounds identification, obtained by chromatographic comparison of their UV spectrum with analytical standards, was followed by quantitative analysis using external standard method. The UV analysis highlighted the presence on HH of two main phytochemicals groups related to caffeoylquinic acid derivatives and naringenin derivatives (Table 1). Due to the absence of commercial standards, caffeoylquinic acid derivatives were expressed as chlorogenic acid, and naringenin derivatives were expressed as naringenin. Caffeoylquinic acid derivatives were the most abundant compounds in samples analyzed whit a concentration of 421 ± 20 mg/L followed by naringenin derivatives that showed a concentration of 27 ± 1 mg/L (Table 1).

Table 1. Phenolic compounds quantified in HH.

Phenolic compounds	HH mg/L ± DS
Caffeoylquinic acid derivatives (£)	421 ± 20
Naringenin derivatives (ø)	27 ± 1

Results are expressed as mean ± standard deviation. (£) Caffeoylquinic acid derivatives were expressed as chlorogenic acid. (ø) Naringenin glycoside derivatives were expressed as naringenin.

Hydrolate of H. italicum stimulate cell viability

MTT assay was performed on HFF1 and SSCs to evaluate the toxicity at different concentrations and timepoints (Fig. 2). The assay was performed after 24h or 48h of culturing under the above described conditions, showing a low toxicity of the treatment at concentrations tested. In the presence of extract, cell viability of SSCs for all the concentration tested was similar to control, represented by untreated cells, after 24h and 48h (Fig. 2b,d). On the other hand, a significant decrease in cell viability could be observed only in fibroblasts treated with 40 % HH for 24h or 48h (Fig. 2a,c) . Such as SSCs, no cytotoxic effects were detected when HFF1 were cultured with the other concentrations (Fig. 2a,c). Fig. 2 also shows that HH was not toxic for both HFF1 and SSCs for all the concentrations under 30 % tested.

Fig. 2. MTT assay of HFF1 (**a**, **c**) and SSCs (**b**, **d**) cultured for 24 and 48 hours in the presence of different concentrations of HH. Cell viability is expressed as percentage of cell viability as compared to untreated cells (Ctrl 100 %). Data are expressed as mean SD referring to the control * $p < 0.05$.

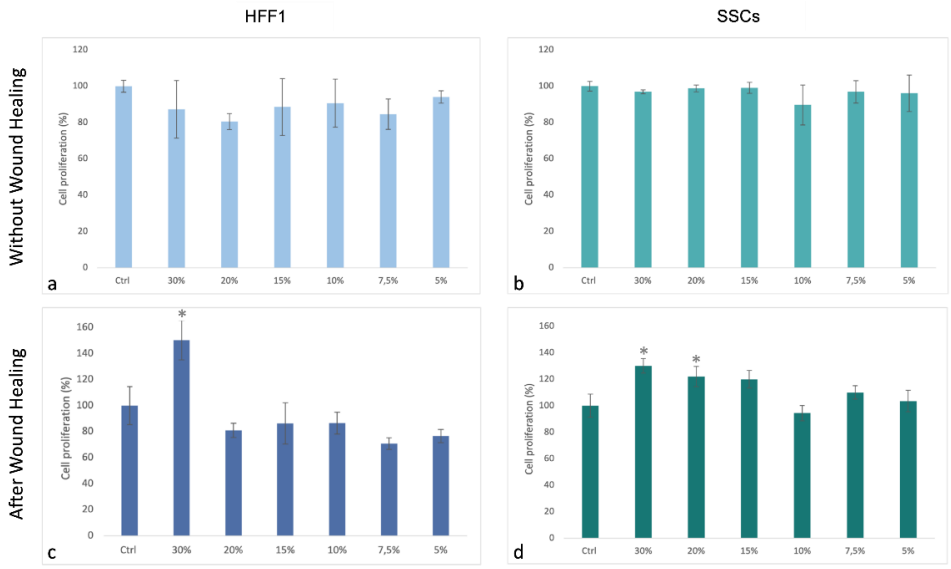

Fig. 3. BrdU assay after 24h of treatment with HH in HFF1 and SSCs before (**a, b**) and after scratch test (**c, d**). Cell viability is expressed as percentage compared to untreated cells (Ctrl 100 %). Data are expressed as mean SD referring to the control * $p < 0.05$.

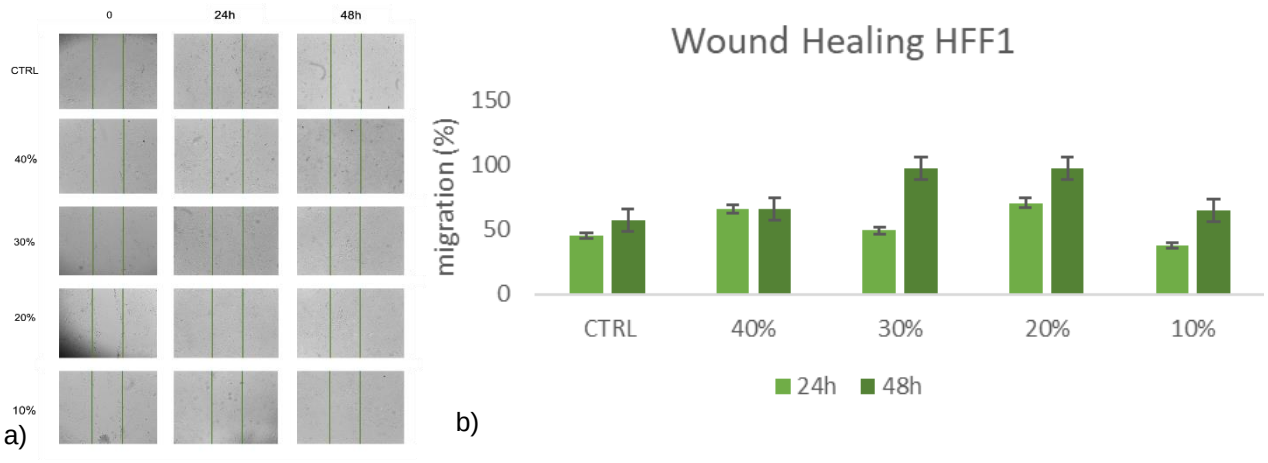

Fig. 4. Fibroblast migration and proliferation after scratch and treatment with different scalar dilutions of HH (40%-10%). Images were taken with inverted light microscope at the time of cutting (day 0), after 24h and after 48h of treatment. Fig. 4b) shows a percentage of wound closure after 24h and 48h compared to untreted cells (Ctrl)

Hydrolate of H. italicum induces cell proliferation

The BrdU assay was performed to analyze cell proliferation (Fig. 3). The test revealed that the treatment with HH does not affect the proliferation of the cell populations analyzed, not undergoing wound. Fig. 3 shows that these cells reached a proliferation rate similar to that observed for the control sample (Fig. 3a,b). On the other hand, after scratch test, a significant increase in cell proliferation for both fibroblasts and stem cells could be observed (Fig. 3c,d). Both cell types show a significant proliferation increase when cultured with 30 % HH for 24h (Fig. 3c,d). While SSCs show a significant increase even after culturing with 20 % HH for 24 h (Fig. 3d).

Hydrolate of H. italicum promotes cell migration

The ability of HH to stimulate fibroblast and stem cells proliferation and/or migration was detected by the scratch assay. Fig. 4 shows fibroblasts treated with the different concentrations of HH (40 %, 30 %, 20 % and 10 %). Figures 4 and 5 show the migration of cells after scratch for different time point (day 0, 24h and 48h). Fig. 4 revealed that fibroblasts treated with 20 % and 30 % HH for 24 h increased the number of migrating cells detectable in the wound site compared to control cells (untreated samples). After 48 hours, samples treated with the other concentrations of HH selected (30 % and 20 %) reached confluence. Fig. 4 show that both samples treated with 30 % and 20 % of HH showed an increased number of migrating cells detectable in the wound site as compared to control untreated cells. In contrast, in the untreated control, the wound site was not healed. On the other hand, cells treated with 10 % of HH showed reduced migration

and proliferation activity, compared to cells treated with 20 % or 30 %. However, fibroblasts treated with 40 % HH were not committed to migration (Fig. 4). Fig. 5 shows SSCs migration and proliferation were induced as early as 24h by 20 % and 10 % HH concentrations. After 48h, also the cells treated with 30 % HH showed complete confluence of the wound site (Fig. 5). Results were also analyzed comparing wound closure area after 24h and 48h of HH treatment to untreated cells, and measured in percentage considering as 0 % the wound area at T0 (moment of wound creation) and 100 % the full confluence of cells in wound area (Fig. 4, panel a and Fig. 5b).

Hydrolate of H. italicum induce a molecular program of skin regeneration

Fig. 6 shows HFF1 cultured in the presence of different concentrations of HH. Results show a modulation of the expression of the TNF-α gene, involved in acute inflammation. In particular, we detected also a significant increase of TNF-α gene expression for samples cultured with 40 % HH, while the concentration of 30 % HH induced a downregulation as compared to control sample (Fig. 6a). Moreover, all treatments induced increased expression of CASP8 and HAS2 genes, (Fig. 6b,c), being particularly evident for cells cultured in the presence of 20-30 % of HH. Fig. 7 shows gene expression analysis of SSCs. Cells show a significant increase in SOX2 (Fig. 7a), Oct-4 (Fig. 7b) and NANOG (Fig. 7c) expression, when cultured in the presence of 10 %, 20 % and 30 % HH respectively, as compared to control cells.

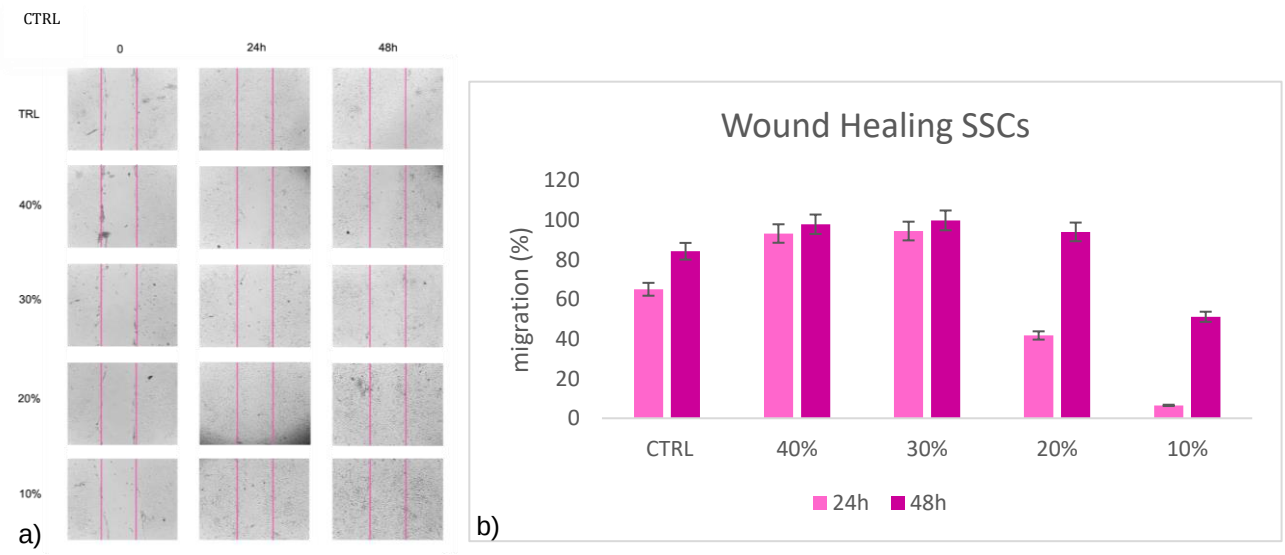

Fig. 5. Stem cells migration and proliferation after scratch and treatment with different scalar dilutions of HH (40%-10%). Images were taken with inverted light microscope at the time of cutting (day 0), after 24h and after 48h of treatment. Fig. 5b) shows a percentage of wound closure after 24h and 48h compared to untreated cells (Ctrl).

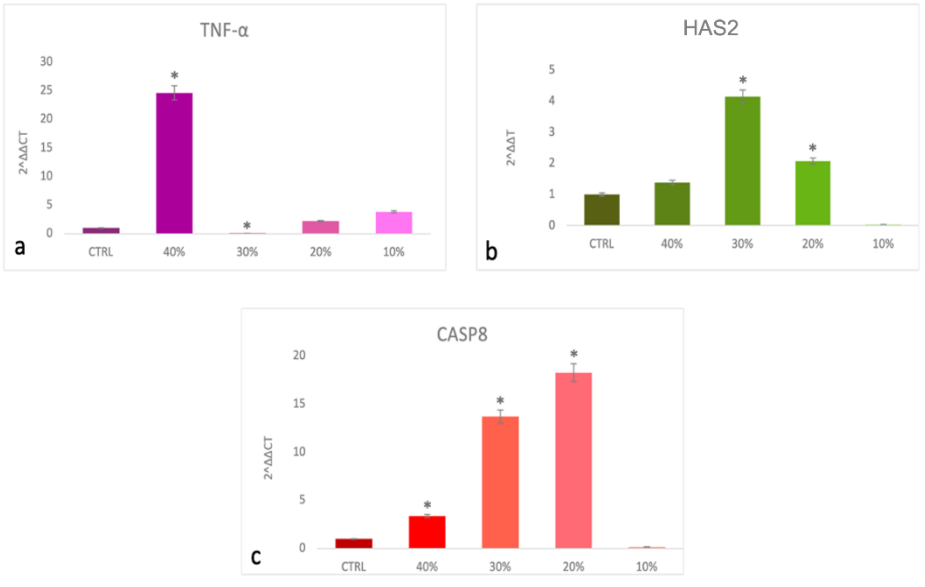

Fig. 6. Gene expression analysis of TNF- α (**a**), HAS2 (**b**) and CASP8 (**c**) in HFF1 cultured in the presence of the different concentrations of HH after the scratch. The expression of each gene was normalized to HPRT1 and plotted as fold of change ($2^{-\Delta\Delta CT}$) relative to the mRNA expression of untreated control (Ctrl), * p value ≤ 0.05.

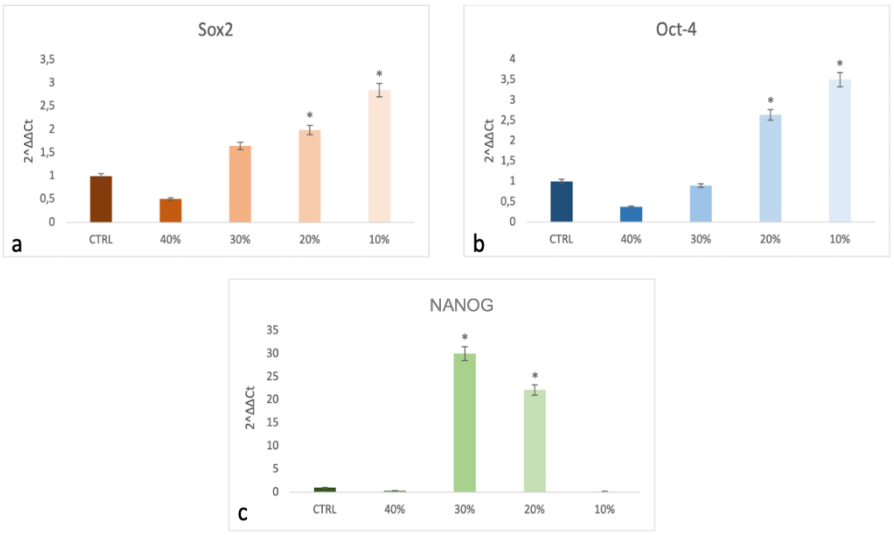

Fig. 7. Gene expression analysis of SOX2 (**a**), Oct-4 (**b**) and NANOG (**c**) in SSCs cultured in the presence of the different concentrations of HH after the scratch. The expression of each gene was normalized to HPRT1 and plotted as fold of change ($2^{-\Delta\Delta Ct}$) relative to the mRNA expression of untreated control (Ctrl), * p value ≤ 0.05.

Discussion

The use of natural products is arising as a novel strategy to enhance tissue regeneration and wound treatments, for their wide availability and fewer side effects [19,20,21]. Accelerate wound healing is one of the main difficult goal in wound repair process [4] that could be influenced by several factors [7,8]. *H. italicum* is a promising ally for its bioactive molecules, already described for antimicrobial and anti-inflammatory properties [28]. Here we analyzed the effect of hydrolate of *H.italicum (HH)* in wound healing. The phytochemical assay revealed that HH is reach in phenolic compounds (Table 1), already known for their role in wound healing by preventing and protecting against radical oxidative damage. Within this context, it has been previously shown that also flavonoids as naringenin and chlorogenic acid, are able to accelerate cell migration and improve wound closure [29]. Analysis performed on the HH tested in this study shows that caffeoylquinic acid derivatives (chlorogenic acid) were the most abundant compounds followed by naringenin derivatives (Table 1). Chlorogenic acid regulates collagen secretion in the final phase of wound healing for the formation of scar tissue [29,30,31]. Available evidence shows that naringenin has important pharmacological properties including anti-inflammatory, antioxidant, neuroprotective, hepatoprotective, and anticancer activities [32]. Our results fit perfectly with these previous findings, since hydrolate of *Helichrysum italicum* can promote regenerative processes without showing a cytotoxic effect on cell proliferation, especially at low concentrations (Figs. 2, 3). The major markers of stemness and wound- related genes SOX2, Oct-4 and NANOG (Fig. 7a,b,c) and inflammation-related genes TNF-α, HAS2, CASP8 (Fig. 6, panels a, and c) are expressed by SSCs and HFF1 and following implementation of "wound healing" showed that concentrations of 20 % and 30 % were the most suitable. Stem cells cultured in the presence of 20 % or 30 % HH, show maintenance of pluripotency, as assessed by the expression of SOX2, Oct-4, and NANOG genes (Fig. 7a,b,c). These results are further inferred by the "wound healing" assay, in which a good regenerative ability of HH was observed in the presence of wounds for concentrations not exceeding 30 % (Figs. 4, 5). The importance of the treatment lies in the ability of this extract to stimulate cell proliferation only in the presence of damage as shown by the BrdU assay (Fig. 3c,d) and by the wound healing assay (Figs. 4, 5). This is extremely important as inappropriate stimulation at any time could adversely affect it, leading to uncontrolled cell proliferation and tumorigenic mechanisms. Many studies show that using the rich chemical composition of plants provides different drugs for clinical application [33]. In the present study we investigate the activity of hydrolate *of H. italicum* on different skin cell populations, highlighting its regenerative effect on tissue regeration. The data suggest that hydrolate of *H.italicum* has therapeutic potential. Only few studies on the use of *Helichrysum italicum* in wound treatment have been performed *in vivo*. Noteworthy other authors describe that 4 patients with wounds, visited in a dermatology clinic, and all showed good healing following treatment with *H. italicum* [34]. No secondary infection or irritation was observed in the wound area, demonstrating the antimicrobial, anti-inflammatory and regenerative benefits of *Helichrysum italicum* [34]. Hydrolate of *H.italicum* could be combined with nanomaterials that can release the extract in situ during the healing time to

stimulate cell proliferation after injury. Nanomaterials could be applied to the damaged area and control the release of the extract. For example, a gel formulation containing *Helichrysum italicum* loaded in chitosan nanoparticles (NPs) was developed for dermatological applications [35]. This formulation presented pH properties, viscosity and spread ability suitable for topical application. Finally, the performance of the gel in topical application to the skin of volunteers was evaluated by noninvasive methods [35]. These assays demonstrated that the properties of the thermal water-based gel formulation developed with NPs of chitosan loaded *with H. italicum* can improve skin hydration and maintain skin function. There are new ideas for *in silico* studies of the molecular mechanisms of the bioactive polyphenols in *Helichrysum italicum*, along with new suggestions for improving their bioavailability through different encapsulation techniques [36]. A study was performed to investigate the dermo protective activity of cotton gauze and polypropylene nonwoven fabrics impregnated with *H. italicum* by the combined supercritical CO_2 extraction and solvent impregnation [37]. Results of *in vivo* studies in human volunteers confirmed the suitability of the active components of *H. italicum* to maintain healthy skin [37]. Further in-depth studies are needed to evaluate other properties and effect of all the natural biomolecules recovered in HH, on the skin. In conclusion our results indicate that hydrolate of *Helichrysum italicum* could be a good candidate topic treatment of skin wounds, and pave the way for future *in vivo* studies.

Authors contribution statement

Conceptualization, D.S., E.B., E.A., A.P., A.N. and M.M.; data curation, D.S., E.B., G.G., S.C.; formal analysis D.S., E.B., G.G and S.C.; investigation, D.S., E.B., G.G. and M.M.; methodology, G.G., D.S., R.S., M.A.M., G.S., D.D. and M.F.; supervision, M.M.; validation, D.S., E.B.; writing–original draft, D.S.; writing—review and editing, S.C., E.A., A.N. and M.M. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

There is no conflict of interest.

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Informed consent was obtained from all patients in accordance with the EU and Italian ethical and medical regulations (Ethical Clearance N. 0021565/2018, 22/03/2018-Commissione Etica CNR).

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