



# Wound-Healing Potential of a Novel *Helichrysum italicum* Film-Forming Gel: Enhanced Bioactivity Through Sustained Delivery and Fibroblast Modulation

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## Abstract

**Purpose** Wound healing is a highly orchestrated process that can be impaired under pathological conditions, highlighting the need for advanced topical formulations. This study aimed to comparatively evaluate *Helichrysum italicum* essential oil (EO) and a novel film-forming gel (FFG) incorporating this EO in an in vitro fibroblast-based wound model.

**Methods** Formulations were prepared using polyvinyl alcohol, carboxymethyl cellulose, glycerol, polysorbate 80, and ethanol, yielding stable and skin-compatible gels. Chemical analyses (GC–MS and total phenolic content) verified the presence of bioactive sesquiterpenes and phenolic compounds, while physical characterization confirmed appropriate pH, viscosity, and homogeneity. Biological assays revealed a biphasic, dose-dependent effect of EO: low-to-intermediate concentrations (250–500 µg/mL) enhanced fibroblast viability and proliferation, whereas a high concentration (2 mg/mL) induced cytotoxicity. Additionally, antioxidant, molecular (immunocytochemistry, qRT-PCR, Western blotting), and collagenase inhibition assays were conducted.

**Results** Among the gel formulations, F2 (0.5% w/w EO) exhibited the most favorable profile, significantly improving cell viability, proliferation, and migration compared to EO alone. Antioxidant analyses showed that F2 markedly reduced intracellular superoxide production and lipid peroxidation while increasing glutathione levels. Molecular evaluations demonstrated significant upregulation of extracellular matrix-related markers (COL1A1, FN1, and MMP7), indicating coordinated ECM synthesis and remodeling. F2 also inhibited collagenase activity, supporting the preservation of structural proteins.

**Conclusion** Incorporation of *H. italicum* EO into a film-forming gel enhanced bioactivity, likely via improved local retention and a protective milieu. Although confined to an in vitro model and single polymer, these findings warrant further preclinical investigation of EO-loaded FFGs in wound healing.

**Keywords** *Helichrysum italicum* · Film-forming gel · Wound healing · Fibroblast · Extracellular matrix

## Introduction

The healing of wounds represents a fundamental physiological response triggered by a disruption in the structural and functional integrity of living tissues [1]. This dynamic and highly coordinated process, essential for the restoration of the skin's critical barrier function, involves a complex cascade of overlapping phases [2]. These stages are governed by an intricate interplay of various cells, including keratinocytes, fibroblasts, and immune cells, alongside a precise balance of cytokines, growth factors, and enzymes [3]. However, this delicate process can be significantly impaired by factors such as infection or underlying disease states, leading to the development of chronic, non-healing

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wounds that pose a substantial clinical and economic burden globally [4]. Consequently, research efforts are increasingly focused on identifying therapeutic agents capable of effectively modulating the healing cascade. Given the considerable complexity of the wound healing process, which involves numerous distinct pathways, there is a clear rationale for utilizing multi-functional compounds from natural sources that can modulate several targets simultaneously.

The development of effective topical formulations for wound management often faces challenges associated with conventional semi-solid dosage forms, including short residence time and poor adherence due to accidental removal [5]. In response to these limitations, film-forming systems have emerged as an innovative strategy in pharmaceutical technology. A specific and promising embodiment of this approach is the film-forming gel, a semi-solid formulation that undergoes a phase transition upon application [6]. Following solvent evaporation, the gel transforms in situ into a thin, translucent, and adherent film that conforms to the contours of the skin. This resulting film acts as a protective barrier, shielding the wound from external contaminants and mechanical stress while simultaneously facilitating sustained drug delivery [7]. The performance of these systems, including their adhesion, flexibility, and drug release profile, is predominantly governed by the properties of the polymeric excipients used, which must be biocompatible and possess optimal film-forming [8]. Consequently, film-forming gel formulations represent a versatile and patient-friendly platform that combines the easy application of a gel with the prolonged therapeutic benefits of a protective film.

The search for effective wound healing agents has increasingly turned towards natural extracts, valued for their biocompatibility, biodegradability, and multi-faceted therapeutic activities [9]. Among these, *H. italicum* (Immortelle), a plant indigenous to the Mediterranean region, has garnered significant attention for its historical use in treating skin ailments and its promising biological properties [10]. The therapeutic potential of *H. italicum* is largely attributed to its rich and diverse phytochemical composition, which includes compounds such as neryl acetate,  $\alpha$ -pinene, and caffeoylquinic acid derivatives [11]. These constituents are known to confer potent antioxidant, anti-inflammatory, and antimicrobial activities, which are critically important across the sequential phases of wound healing [12]. Furthermore, research indicates that extracts from this plant can modulate key inflammatory markers, such as reducing IL-1 $\beta$  levels, thereby creating a more conducive environment for tissue repair [13]. A notable advantage of *H. italicum*-based products, including its essential oil and hydrolates, is their favorable safety profile and lack of skin irritation, as documented in preclinical toxicity assessments

[14]. These properties make them compelling candidates for development into novel wound healing therapies. Notably, while the pure essential oil exhibits concentration-dependent cytotoxicity at high doses, its incorporation into appropriately formulated topical products, including gels and ointments, has been shown to maintain efficacy while ensuring dermal tolerability [10, 12].

Given the established wound-healing properties of *H. italicum* and the advantages offered by advanced delivery systems, this study aimed to directly compare the efficacy of *H. italicum* essential oil with a novel *H. italicum*-loaded film-forming gel formulation in an in vitro model utilizing dermal fibroblasts. While previous research has explored *H. italicum* in conventional vehicles such as ointments and hydrogels, our film-forming gel (FFG) represented a conceptually distinct platform. Unlike traditional semi-solid formulations that can be easily rubbed off, this FFG underwent a phase transition upon application, forming a thin, adherent, and protective film in situ. This unique mechanism was designed to prolong skin residence time, provide a moist wound-healing environment, and facilitate sustained release of the active compounds. We hypothesized that this film-forming gel would enhance the bioactivity of the extract by providing a sustained release and a protective microenvironment, thereby more effectively promoting cellular processes critical for wound repair. To test this hypothesis, we evaluated key parameters of dermal fibroblast functions relevant to wound healing, including cell migration via a scratch assay and the expression of pivotal extracellular matrix (ECM) proteins—specifically fibronectin 1 (FN1), collagen type 1 alpha 1 (COL1A1), and matrix metalloproteinase 7 (MMP7)—using gene expression analysis and immunoassays. This work represented, to our knowledge, the first comparative investigation of these two formulations of *H. italicum*, seeking to elucidate their potential mechanisms in modulating ECM reorganization and fibroblast function to advance topical wound therapy development.

## Materials and Methods

### Materials

The essential oil of *H. italicum* (Roth) G. Don was commercially supplied by Botanika Tarım (Antalya, Türkiye; Batch #HI-EO-2023-11). For film-forming gel preparation: Polyvinyl alcohol (PVA 1788, MW 88,000–97,000, 87–89% hydrolyzed), carboxymethyl cellulose sodium salt (CMC, low viscosity), ethanol ( $\geq 99.8\%$ ), glycerol ( $\geq 99.5\%$ ), and polysorbate 80 (Tween<sup>®</sup> 80) were all purchased from Merck KGaA (Darmstadt, Germany; Cat.

#108630, #21902, #100983, #104094, #817061 respectively). For total phenolic content determination: Gallic acid ( $\geq 98\%$ , #G7384), sodium carbonate ( $\geq 99.5\%$ , #S7795), and Folin-Ciocalteu reagent (#F9252) were obtained from Sigma-Aldrich (St. Louis, MO, USA). For anticollagenase assays: Collagenase Type I from *Clostridium histolyticum* ( $\geq 125$  CDU/mg, #C0130), dimethyl sulfoxide (DMSO,  $\geq 99.9\%$ , #D8418), Tricine ( $\geq 99\%$ , #T0377), and N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA, #F5135) were supplied by Sigma-Aldrich (St. Louis, MO, USA). For cell culture: Dulbecco's Modified Eagle Medium (DMEM, #11965092), fetal bovine serum (FBS, #16000044), and penicillin-streptomycin (10,000 U/mL, #15140122) were from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Human dermal fibroblasts (HDFa, ATCC<sup>®</sup> PCS-201-012<sup>™</sup>, Lot #70025387) were acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were used between passages 3–6 for all experiments. Cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose, GlutaMAX<sup>™</sup> Supplement, Gibco, #10566016) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, #F2442), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (Sigma-Aldrich, #P4333). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Mycoplasma contamination was routinely tested using a PCR-based kit (Venor<sup>™</sup> GeM Classic, Minerva Biolabs).

## Methods

### Formulation and Evaluation of Film-forming-gels Containing *H. Italicum* Essential Oil

**Formulation of Film-forming-gels Containing *H. Italicum* Essential Oil** Polyvinyl alcohol (PVA 1788, MW 88,000–97,000, 87–89% hydrolyzed), carboxymethyl cellulose (CMC), glycerin, active compound (*H. italicum* essential oil), Tween 80, ethanol and purified water were used for film-forming-gels preparation. The aqueous solution containing 5% by weight (w/w) Polyvinyl alcohol (PVA 1788) was dispersed for 24 h and then heated at 95 °C for 1 h to dissolve completely. Then, 1% (w/w) Tween 80, 5% (w/w) glycerol and 0.8% (w/w) sodium carboxymethyl cellulose were added to this mixture, which came to room temperature, and stirred for 2 h. Finally, 25% by weight

(w/w) ethanol and different concentrations (0.0, 0.25, 0.5, and 1.0% w/w) of *H. italicum* essential oil were added to the polymer solution, and the resulting mixture was stirred for 30 min (Table 1). At the end of this period, the film-forming gel formulations containing *H. italicum* essential oil were stored at + 4 °C, away from light [15]. Ethanol was used solely as a solvent to facilitate the incorporation of the lipophilic essential oil into the aqueous polymer blend and was not used for the extraction of the plant material itself.

**Physical Study of Film-forming-gels Formulations** The prepared film-forming gels were characterized for their organoleptic properties, including color, odor, homogeneity, and physical appearance. The pH of each formulation was measured in triplicate at room temperature using a digital pH meter (Thermo Scientific ORION STAR A215). Viscosity analysis was performed with a Fungilab Expert Viscometer equipped with an L4 spindle rotating at 100 RPM, also conducted at room temperature.

### Chemical study of film-forming-gels containing *H. Italicum* essential oil

**Volatile Organic Compounds of *H. Italicum* Essential Oil** *H. italicum* essential oil (5 mg) was dissolved in 10 ml of ethanol and then filtered through 0.45  $\mu$ m nylon filters. Then, 1  $\mu$ L of this solution was injected into the GC-MS instrument equipped with an HP-5 capillary column (30 m  $\times$  0.32 mm  $\times$  0.25  $\mu$ m) in splitless mode at a constant helium flow of 1.0 ml/min. The oven temperature program was initially held at 60 °C for 10 min, then increased by 20 °C min<sup>-1</sup> to 250 °C and held at this temperature for 11 min. Wiley and NIST (Wiley Mass Spectral Data Recording, 7th Edition, NIST 98 Library) were used as libraries [16].

**Determination of total phenolic contents (TPC) of film-forming-gels containing *H. italicum* essential oil and *H. italicum* essential oil** TPC of both the *H. italicum* essential oil and the film-forming gel formulations was quantified according to the Folin-Ciocalteu colorimetric method, adapted from Shen et al. with minor modifications [17]. In brief, 1 g of each sample was dissolved in 10 mL of ethanol. From this stock solution, a 10  $\mu$ L aliquot was combined with 150  $\mu$ L of a 6% Folin-Ciocalteu reagent and allowed

**Table 1** The components of film-forming gels formulations (w/w)

| No        | Polyvinyl alcohol 1788 | Sodium carboxymethyl cellulose | Glycerol | Polysorbate 80 | Ethanol | Water | <i>H. italicum</i> essential oil |
|-----------|------------------------|--------------------------------|----------|----------------|---------|-------|----------------------------------|
| <b>F0</b> | 5.0                    | 0.8                            | 5.0      | 1.0            | 25      | 63.20 | -                                |
| <b>F1</b> | 5.0                    | 0.8                            | 5.0      | 1.0            | 25      | 62.95 | 0.25                             |
| <b>F2</b> | 5.0                    | 0.8                            | 5.0      | 1.0            | 25      | 62.70 | 0.5                              |
| <b>F3</b> | 5.0                    | 0.8                            | 5.0      | 1.0            | 25      | 62.20 | 1                                |

to equilibrate for 3 min. Subsequently, 50  $\mu\text{L}$  of sodium carbonate (0.6 M) was added to the mixture.

The reaction was allowed to proceed for 2 h at ambient temperature ( $25 \pm 2$  °C) under light-protected conditions. Following this incubation, the absorbance was measured at 740 nm using a Shimadzu UV 1800 spectrophotometer. The TPC was determined from a gallic acid standard curve prepared in methanol and is expressed as milligrams of gallic acid equivalents (GAE) per gram of sample (Fig. S2). Although essential oils are primarily composed of terpenoids, the Folin–Ciocalteu assay detects total reducing capacity rather than strictly phenolic compounds. Therefore, in the context of essential oils, TPC values could be interpreted as an index of overall reducing/antioxidant potential rather than a direct quantification of classical phenolic molecules. This approach is widely used in essential oil research as a comparative antioxidant proxy.

#### Biological studies of film-forming-gels containing *H. italicum* essential oil

**In Vitro anticollagenase activities of film-forming-gels containing *H. italicum* essential oil and *H. italicum* essential oil** The inhibitory activity of the film-forming gel formulations against collagenase type I was evaluated spectrophotometrically, employing a modified method based on Thring et al. stock solutions of the formulations were prepared in dimethyl sulfoxide (DMSO) at specified concentrations [18]. The reaction mixture for the test samples was assembled by combining 50  $\mu\text{L}$  of collagenase type I solution (0.8 U/mL) with 50  $\mu\text{L}$  of the DMSO-dissolved gel formulations. Subsequently, 0.9 mL of Tricine buffer (pH 7.5) was added.

For comparative purposes, the approximate final concentrations of *H. italicum* EO in the culture medium for the gel formulations were calculated based on the initial oil loading and the dilution factor in the experimental setup. Specifically, treatment with the F1 (0.25% w/w), F2 (0.5% w/w), and F3 (1.0% w/w) formulations resulted in nominal in-well EO concentrations of approximately 125  $\mu\text{g/mL}$ , 250  $\mu\text{g/mL}$ , and 500  $\mu\text{g/mL}$ , respectively. These values align directly with the concentration range tested for the free EO, allowing for a more intuitive comparison of their biological effects.

A control solution, containing no gel formulation, and a blank solution, where the enzyme was replaced with an equivalent volume of DMSO, were prepared in parallel. All solutions (blank, control, and samples) underwent an initial 30-minute incubation at 25 °C. Following this, 0.05 mL of the 1 mM FALGPA substrate solution was added to each,

initiating a second incubation period of 15 min at 25 °C. The absorbance was then measured at 340 nm using a Shimadzu UV 1800 spectrophotometer, with sample and control readings taken against the blank.

#### Cell Culture Assay

**Treatment Preparation and Cell Viability Assay** Prior to each experiment, working solutions of *H. italicum* essential oil and the film-forming gel formulations (F0, F1, F2, F3) were freshly prepared and sterilized by filtration. Dermal fibroblasts were plated in 96-well plates at a seeding density of  $5 \times 10^3$  cells per well and incubated for 24 h to allow for adhesion and to achieve approximately 80% confluency. Following this attachment period, the cells were exposed to a range of concentrations of the essential oil (50  $\mu\text{g/mL}$  to 2 mg/mL) and the various gel formulations for durations of 24, 48, and 72 h. For cell treatment, the gel formulations were applied as liquids directly into the cell culture medium at the specified final concentrations. The gelation and film-formation process intended to occur upon application to a surface; in the aqueous environment of the cell culture medium, the formulation components disperse, allowing for cellular exposure to the active compounds.

To determine cellular viability, the Cell Counting Kit-8 (CCK-8) assay was employed. After the respective treatment intervals, the cultures were incubated with 10  $\mu\text{L}$  of the CCK-8 reagent per well for one hour at 37 °C. The absorbance of the resulting solutions was then quantified at a wavelength of 450 nm using a full-wavelength microplate reader (BioTek).

**Proliferation Assay** The assessment of cell proliferation was carried out using a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay (Sigma-Aldrich, #B5002). Based on the initial CCK-8 dose-response findings, specific concentrations of the essential oil and film-forming gel formulations were selected for treatment. Cells were plated at a density of  $5 \times 10^4$  cells per well in 12-well plates and subsequently exposed to these selected concentrations. Following a 24-hour treatment period, DNA synthesis rates were quantified colorimetrically as per the manufacturer's protocol, with absorbance measured at 450 nm.

While the CCK-8 assay provides a measure of metabolically active cells (reflecting both viability and proliferation), it does not specifically quantify de novo DNA synthesis. Therefore, the BrdU incorporation assay was employed as a complementary method to directly assess the fraction of cells entering S-phase, providing a more specific readout of proliferative activity.

**Measurement of Intracellular Superoxide Anion** Intracellular superoxide anion ( $O_2^-$ ) production was measured fluorometrically using dihydroethidium (DHE). The experimental procedure commenced with the plating of cells in 96-well plates at a density of  $5 \times 10^3$  cells per well, which were then given 24 h to adhere. Following this attachment period, the culture medium was aspirated and replaced with fresh medium containing the designated concentrations of *H. italicum* essential oil or the film-forming gel formulations for a 24-hour incubation [Based on the CCK-8 and BrdU results, subsequent mechanistic studies were performed using 500  $\mu\text{g/mL}$  *H. italicum* essential oil and the F2 film-forming gel formulation (0.5% w/w EO). The F0 formulation (0% EO) was included as vehicle control.]. After treatment, the cells were subjected to three washes with phosphate-buffered saline (PBS, pH 7.4) to ensure the removal of any residual compounds. The washed cell monolayers were subsequently incubated with a 10  $\mu\text{M}$  DHE solution for 30 min at 37 °C under light-protected conditions. Upon completion of this incubation, the DHE solution was removed, and a final PBS rinse was performed. Cellular fluorescence was visualized using an Olympus FV3000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan) equipped with a 20 $\times$ /0.75 NA UPlanSApo objective. DHE was excited at 488 nm and emission was collected at 590–620 nm. Quantitative analysis was performed on five randomly selected fields per well using ImageJ software (NIH, Bethesda, MD, USA).

**Immunocytochemical Analysis** Following a 24-hour treatment period with either 500  $\mu\text{g/mL}$  *H. italicum* essential oil, F2 formulation, or F0 vehicle control, following fixation with 4% paraformaldehyde for 12 min at room temperature, the cells were subjected to three 5-minute washes with PBS. A permeabilization step was then performed using 0.1% Triton X-100 in PBS for 15 min. To minimize non-specific antibody binding, a blocking solution of 1% bovine serum albumin (BSA) in PBS was applied for one hour at ambient temperature. The samples were subsequently incubated for one hour at 37 °C with primary antibodies diluted in 1% BSA/PBS, specifically: anti-FN1 (Cell Signaling Technology, #26836; 1:300), anti-COL1A1 (Cell Signaling Technology, #72026; 1:300), and anti-MMP7 (Thermo Fisher Scientific, PA5-87486; 1:400). After thorough washing to remove unbound primary antibodies, the cells were exposed to an HRP-conjugated goat anti-rat IgG secondary antibody (Thermo Fisher Scientific, #31629) at a concentration of 1  $\mu\text{g/mL}$  for one hour at room temperature. Immunofluorescence images were acquired using the same Olympus FV3000 confocal microscope with 40 $\times$ /1.30 NA oil immersion objective. Alexa Fluor 488 (FN1, COL1A1) and Alexa Fluor 568 (MMP7) were excited at 488 nm and 561 nm

respectively. To evaluate the relative expression levels of the target proteins, the area percentage of positively stained cells was quantified using ImageJ software. The number of immunopositive cells was counted manually in at least five random fields per coverslip, and data were expressed as a percentage of total cells.

**Cell Migration Assessment by Scratch Assay** A scratch wound assay was employed to assess cellular migratory capacity. Cells were first cultured in 12-well plates at a density of  $5 \times 10^4$  cells per well until they formed a confluent monolayer. A linear scratch wound was then introduced into each monolayer using a sterile 200  $\mu\text{L}$  pipette tip. Following the removal of dislodged cells and medium by aspiration, the monolayer was rinsed twice with PBS to clear any residual debris. To minimize the contribution of proliferation to wound closure, cells were serum-starved (0.5% FBS) during the 24-hour migration period. This approach reduces proliferation while preserving migratory capacity.

Fresh medium containing either 500  $\mu\text{g/mL}$  *H. italicum* essential oil, F2 formulation, or F0 vehicle control was immediately added to the wells. Wound closure was monitored using an Olympus CKX53 inverted phase-contrast microscope (Olympus Corporation, Tokyo, Japan) equipped with an Olympus DP28 digital camera. Images were captured at 40 $\times$  total magnification at 0 and 24 h post-scratch. Scratch areas were measured using the MRI Wound Healing Tool plugin in ImageJ.

**Quantitative real-time PCR (qRT-PCR) and Western Blot analyses** Total RNA was isolated from cultured fibroblasts using TRIzol™ reagent (Thermo Fisher, #15596026) according to manufacturer's instructions. RNA concentration and purity ( $A_{260}/A_{280} \geq 1.9$ ) were assessed using NanoDrop™ One<sup>®</sup> spectrophotometer (Thermo Fisher). For each sample, 1  $\mu\text{g}$  of total RNA was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814) with random hexamers in a total volume of 20  $\mu\text{L}$ . Thermal cycling conditions were: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min. Quantitative real-time PCR was performed on a QuantStudio™ 3 System (Applied Biosystems) using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, #A25742). Each 20  $\mu\text{L}$  reaction contained 10  $\mu\text{L}$  SYBR Green Master Mix, 0.5  $\mu\text{L}$  each of forward and reverse primers (10  $\mu\text{M}$ ), 2  $\mu\text{L}$  cDNA (diluted 1:10), and 7  $\mu\text{L}$  nuclease-free water. Cycling conditions: 95 °C for 20 s (initial denaturation), followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. A melt curve analysis was performed at the end of each run (65 °C to 95 °C, increment 0.5 °C/5 s) to verify single product amplification and absence of primer-dimers. All reactions were run

in triplicate. Relative gene expression was calculated using the  $2^{-\Delta\Delta C_t}$  method with  $\beta$ -actin as endogenous control and untreated control as calibrator. The corresponding nucleotide sequences for all primers employed are provided subsequently: FN1 forward: 5'-AGG GCA ACG TCT CGG CTA GCC-3' and reverse: 5'-TGC GCA CGG CAG GAT CGT CA-3'; COL1A1 forward: 5'-CGT CGT TAG CGA GAG ACG T-3' and reverse: 5'-GCT GGA GTC ACA GAT TAA-3'; MMP7 forward: 5'-ACG ACT GTA GTA GAC TCG GAT CA-3' and reverse: 5'-CCA TCG ACC TAG AAG CCG AGC AC-3';  $\beta$ -actin forward: 5'-AGT CAG CGA TCT TGA TCA GCC-3' and reverse: 5'-TGC GCT GAG ATG CCG GAC AC-3'.

Protein analysis commenced with the preparation of whole-cell lysates in RIPA buffer, followed by the quantification of total protein content via a Bradford assay. Equal protein aliquots (20  $\mu$ g per lane) were then fractionated under denaturing conditions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were subsequently electroblotted onto PVDF membranes for immunodetection. The membranes were immunoblotted with the following primary antibodies: anti-FN1 (Thermo Fisher, PA5-29578) at a 1:2000 dilution, anti-COL1A1 (Thermo Fisher, PA5-29569) at 1:2000, and anti-MMP7 (Thermo Fisher, PA5-87486) at 1:1000. This was followed by incubation with appropriate HRP-conjugated secondary antibodies. Signal detection was achieved using an enhanced chemiluminescence (ECL) system. For loading control, membranes were stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher, #46430) for 15 min at room temperature and re-probed with anti- $\beta$ -actin antibody (Cell Signaling Technology, #4970, 1:5000 dilution). HRP-conjugated anti-rabbit IgG secondary antibody (Cell Signaling Technology, #7074) was used at 1:3000 dilution. Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature prior to primary antibody incubation. All

primary antibody incubations were performed overnight at 4 °C. Signal detection was performed using Clarity™ Western ECL Substrate (Bio-Rad, #1705061) and images were acquired with ChemiDoc™ MP Imaging System (Bio-Rad).

**Statistical Analysis** The results are presented as mean  $\pm$  standard deviation. Normality of data distribution was confirmed using the Shapiro-Wilk test prior to all statistical analyses. For comparisons across multiple experimental groups, one-way analysis of variance (ANOVA) was employed, with Tukey's honest significant difference test applied for post hoc analysis. In experiments evaluating the effects of multiple factors over time, a two-way ANOVA was utilized, followed by Bonferroni post-test corrections for multiple comparisons. A p-value below 0.05 was established as the threshold for statistical significance in all analyses. All statistical procedures were executed using GraphPad Prism software (version 8.0, GraphPad Software, San Diego, CA).

## Results and Discussion

### Physical Study of Film-forming-gels Formulations Containing *H. Italicum* Essential Oil

Several film-forming gel formulations were successfully prepared using different concentrations of *H. italicum* essential oil (Table 1). All formulations exhibited a homogeneous and smooth texture. Their physical appearance ranged from off-white (F0, F1, F2) to light yellow (F3), depending on the oil concentration. The pH values of the formulations were between 6.12 and 6.26, within the physiological skin pH range. Viscosity values were between 4.061 and  $4.076 \times 10^3$  cP, which is suitable for dermal application (Table 2). Although the measured pH exceeds the canonical acidic pH of healthy stratum corneum, it is comparable to commercially available hydrogel dressings and remains compatible with fibroblast viability. Acidic pH is primarily maintained

**Table 2** Physical study of film-forming gels formulations containing *H. italicum* essential oil

| Parameters                    | Film-forming gels formulations |                                                         |                                                         |                                                         |
|-------------------------------|--------------------------------|---------------------------------------------------------|---------------------------------------------------------|---------------------------------------------------------|
|                               | F0                             | F1                                                      | F2                                                      | F3                                                      |
| Appearance                    | Viscous Liquid                 | Viscous Liquid                                          | Viscous Liquid                                          | Viscous Liquid                                          |
| Color                         | Off white                      | Off white                                               | Off white                                               | Light yellowish                                         |
| Smell                         | Non Scent                      | Characteristic odor of <i>H. italicum</i> essential oil | Characteristic odor of <i>H. italicum</i> essential oil | Characteristic odor of <i>H. italicum</i> essential oil |
| Texture                       | Homogeneous                    | Homogeneous                                             | Homogeneous                                             | Homogeneous                                             |
| pH values                     | 6.26 $\pm$ 0.02                | 6.21 $\pm$ 0.01                                         | 6.16 $\pm$ 0.01                                         | 6.12 $\pm$ 0.02                                         |
| Viscosity ( $\times 10^3$ cP) | 4.0764 $\pm$ 0.037             | 4.0705 $\pm$ 0.013                                      | 4.0676 $\pm$ 0.016                                      | 4.0615 $\pm$ 0.018                                      |
| Separation                    | No                             | No                                                      | No                                                      | No                                                      |

Values expressed are the mean  $\pm$  standard deviation of three measurements

by the intact stratum corneum; in wounded skin where the barrier is disrupted, the surface pH rises toward neutral. Therefore, the formulation pH is not expected to impede dermal fibroblast functions relevant to wound healing. Viscosity values were between  $4.061$  and  $4.076 \times 10^3$  cP, which is suitable for dermal application.

### Volatile Organic Compounds of *H. Italicum* Essential Oil

GC/MS analysis identified fifty-four distinct compounds in the *H. italicum* essential oil (Fig. S1), the composition of which is detailed in Table 3. The predominant constituents were  $\gamma$ -Curcumen (15.563%), (1R)- $\alpha$ -Pinene (13.866%),  $\beta$ -Selinene (8.444%), and Neryl acetate (8.266%), followed by  $\alpha$ -selinene (6.270%), Di-epi- $\alpha$ -cedrene-(I) (5.267%),  $\beta$ -Caryophyllene (4.377%), DL-Limonene (4.307%), Geranyl propionate (2.900%), (-)- $\alpha$ -Copaene (2.864%), and (Z, E)- $\alpha$ -Farnesene (2.811%).

### Determination of TPC of Film-forming-gels Formulations Containing *H. Italicum* Essential Oil and *H. Italicum* Essential Oil

TPC of the film-forming gel formulations, as quantified by the Folin-Ciocalteu method, was determined to be between  $9.25 \pm 0.025$  and  $35.60 \pm 0.115$  mg GAE/g (Table 4). In contrast, the *H. italicum* essential oil itself exhibited a significantly higher TPC of  $124.60 \pm 0.20$  mg GAE/g. The TPC was determined from a gallic acid standard curve prepared in methanol and is expressed as milligrams of GAE per gram of sample (Fig. S2). The substantial reduction in TPC in gel formulations ( $9.25$ – $35.60$  mg GAE/g) compared to pure essential oil ( $124.60$  mg GAE/g) is primarily attributable to dilution. The film-forming gel matrix contains only  $0.25$ – $1.025.0\%$  w/w essential oil, and the polymeric components (PVA, CMC) do not contribute to phenolic content. Additionally, some phenolic compounds may interact with the polymer matrix or undergo partial degradation during formulation, though the retention of anticollagenase activity suggests that bioactive constituents remain functional.

### In Vitro Anticollagenase Activities

As presented in Table 5, the  $IC_{50}$  values for the *H. italicum* essential oil and its corresponding film-forming gel formulations against collagenase type I were quantified. The analysis revealed potent anti-collagenase activity for the pure essential oil ( $IC_{50} = 35.73 \pm 0.47$   $\mu$ g/mL). This activity was retained in the film-forming gels, with  $IC_{50}$  values of  $421.814 \pm 0.74$   $\mu$ g/mL (F1),  $377.16 \pm 0.76$   $\mu$ g/mL (F2), and

$237.13 \pm 0.80$   $\mu$ g/mL (F3), demonstrating a concentration-dependent effect (Fig. S3, Fig. S4 and Fig. S5).

### Cytotoxicity and Proliferative Effects of *H. Italicum* Essential Oil and Film-forming Gel Formulations on Dermal Fibroblasts

The evaluation of cellular viability revealed distinct and concentration-dependent effects of *H. italicum* essential oil on dermal fibroblasts over 24, 48, and 72-hour exposures (Fig. 1A). The essential oil exhibited a biphasic response. At lower to intermediate concentrations ( $250$   $\mu$ g/mL and  $500$   $\mu$ g/mL), it significantly enhanced cellular viability. Specifically, treatment with  $250$   $\mu$ g/mL and  $500$   $\mu$ g/mL for 24 h increased viability by 10.3% and 35.7%, respectively ( $n=7$ ,  $p=0.015$  and  $p<0.0001$ ). This promotive effect was sustained at the 48-hour time point, with viability enhancements of 12.4% and 27.1% for the same concentrations ( $p=0.011$  and  $p=0.0001$ ). In contrast, the highest concentration tested ( $2$  mg/mL) demonstrated a time-dependent cytotoxic effect, reducing viability by 10.2%, 17.2%, and 20.2% at 24, 48, and 72 h, respectively ( $p<0.05$  and  $p<0.001$ ). Treatments with  $50$   $\mu$ g/mL,  $100$   $\mu$ g/mL, and  $1$  mg/mL did not significantly alter viability compared to the untreated control at any time point ( $p=0.14$ ).

The film-forming gel formulations displayed a unique profile, further underscoring the benefits of the delivery system (Fig. 1B). The F1 formulation ( $0.25\%$  w/w EO) showed a modest but non-significant trend toward increased viability at 24 h (8.3% increase vs. F0,  $p=0.18$ ), which diminished at 48 h and 72 h (Fig. 1B). The F2 formulation ( $0.5\%$  w/w essential oil) consistently and significantly promoted fibroblast viability, increasing it by 42.1%, 33.9%, and 16.2% at 24, 48, and 72 h, respectively, compared to untreated control ( $p<0.0001$ , and  $p=0.016$ ). The F3 formulation ( $1.0\%$  w/w) exhibited time-dependent cytotoxicity, reducing viability by 7.3% at 24 h ( $p=0.042$ ), 12.1% at 48 h ( $p=0.003$ ), and 18.5% at 72 h ( $p<0.0001$ ) compared to F0. Consistent with these findings, BrdU incorporation was reduced by 8.5% in F3-treated cells ( $p=0.021$ ) and unchanged in F1-treated cells ( $p=0.62$ ) relative to F0 (Fig. 1D).

The proliferative capacity of dermal fibroblasts, as assessed by BrdU incorporation, corroborated the viability findings (Fig. 1C). Treatment with  $250$   $\mu$ g/mL and  $500$   $\mu$ g/mL of the essential oil for 24 h significantly induced cellular proliferation, increasing the proliferation rate by 9.5% and 26.3%, respectively ( $p=0.021$  and  $p=0.0003$ ). This proliferative boost was even more pronounced with the F2 gel formulation, which enhanced proliferation by 40.7% ( $p<0.0001$ , Fig. 1D). In contrast, the highest doses— $2$  mg/mL of essential oil and the F3 gel formulation—inhibited proliferation, reducing it by 13.8% and 8.5%, respectively.

**Table 3** Chemical composition of the *H. italicum* essential oil

| Compound Number | Retention Time (RT) (min) | Compound Name                                                                    | Percentage Distribution |
|-----------------|---------------------------|----------------------------------------------------------------------------------|-------------------------|
| 1               | 6.979                     | (1R)-2,6,6-trimethyl-Bicyclo[3.1.1]hept-2-ene ((1R)- $\alpha$ -Pinene)           | <b>13.866</b>           |
| 2               | 7.528                     | 7,7-dimethyl-2-methylene-Bicyclo[2.2.1]heptane ( $\alpha$ -Fenchene)             | 0.993                   |
| 3               | 11.499                    | 1-methyl-2-(1-methylethyl)-Benzene ( $\alpha$ -Cymene)                           | 0.481                   |
| 4               | 11.625                    | 1-methyl-4-(1-methylethenyl)-Cyclohexene ( <b>DL-Limonene</b> )                  | <b>4.307</b>            |
| 5               | 12.248                    | Isobutyl angelate                                                                | 0.545                   |
| 6               | 12.383                    | 1-methyl-4-(1-methylethyl)-1,4-Cyclohexadiene ( $\gamma$ -Terpinene)             | 0.654                   |
| 7               | 12.97                     | 1-methyl-4-(1-methylethylidene)-Cyclohexene ( $\alpha$ -ERPINOLENE)              | 0.285                   |
| 8               | 13.044                    | 2-dimethylaminoethyl Fumaric acid undecyl ester                                  | 0.114                   |
| 9               | 13.203                    | 3,7-dimethyl-1,6-Octadien-3-ol ( <b>Linalol</b> )                                | 1.252                   |
| 10              | 13.91                     | 5,7-DIBROMO-2-METHYL-8-QUINOLINYL DIETHYLCARBAMATE                               | 0.084                   |
| 11              | 13.971                    | 2-Methylbutyl angelate                                                           | 1.062                   |
| 12              | 14.306                    | 4-methyl-1-(1-methylethyl)-3-Cyclohexen-1-ol ( <b>4-Terpineol</b> )              | 0.260                   |
| 13              | 14.399                    | 3-(Hydroxymethylene)-5 $\alpha$ -cholestan-2 $\beta$ -one                        | 0.496                   |
| 14              | 14.478                    | $\alpha$ - $\alpha$ -0,4-trimethyl-3-Cyclohexene-1-methanol ( <b>Terpineol</b> ) | 0.428                   |
| 15              | 14.599                    | Fluphenazine ( <b>Tensofin</b> )                                                 | 0.071                   |
| 16              | 14.655                    | (Z)-6-undecen-2-ol ( <b>Nostrenol</b> )                                          | 0.066                   |
| 17              | 14.865                    | (Z)-3,7-dimethyl-2,6-Octadien-1-ol ( <b>Nerol</b> )                              | 0.889                   |
| 18              | 15.13                     | 2,3,3-trimethyl-1-Butene ( <b>Triptene</b> )                                     | 0.404                   |
| 19              | 15.381                    | 3-methyl-2-Butenoic acid hexyl ester ( <b>Hexyl Senecioate</b> )                 | 0.273                   |
| 20              | 15.502                    | Tridecane                                                                        | 0.178                   |
| 21              | 16.08                     | (Z)-3,7-dimethyl-2,6-Octadien-1-ol acetate ( <b>Neryl acetate</b> )              | <b>8.266</b>            |
| 22              | 16.187                    | $\alpha$ -Murolene                                                               | 0.366                   |
| 23              | 16.21                     | $\alpha$ -Ylangene                                                               | 0.282                   |
| 24              | 16.257                    | (-)- $\alpha$ -Copaene                                                           | <b>2.864</b>            |
| 25              | 16.308                    | Tetradecane                                                                      | 0.151                   |
| 26              | 16.499                    | Di-epi- $\alpha$ -cedrene-(I)                                                    | <b>5.267</b>            |
| 27              | 16.624                    | $\beta$ -Caryophyllene                                                           | <b>4.377</b>            |
| 28              | 16.662                    | (Z,E)-3,7,11-trimethyl-1,3,6,10-Dodecatetraene ((Z,E)- $\alpha$ -Farnesene)      | <b>2.811</b>            |
| 29              | 16.718                    | (E)-3,7-dimethyl-2,6-Octadien-1-ol propanoate ( <b>Geranyl propionate</b> )      | <b>2.900</b>            |
| 30              | 16.755                    | trans- $\beta$ -Farnesene                                                        | 1.135                   |
| 31              | 16.862                    | $\alpha$ -Humulene                                                               | 0.696                   |
| 32              | 16.927                    | 2-Epi- $\alpha$ -Cedrene                                                         | 1.025                   |
| 33              | 16.992                    | $\gamma$ -Curcumene                                                              | 15.563                  |
| 34              | 17.123                    | $\beta$ -Eudesmene ( $\beta$ -Selinene)                                          | 8.444                   |
| 35              | 17.174                    | $\alpha$ -selinene                                                               | 6.270                   |
| 36              | 17.202                    | $\delta$ -Cadinene                                                               | 0.400                   |
| 37              | 17.262                    | (-)-6- $\alpha$ -Cadina-4,9-diene ( $\alpha$ -Amorphene)                         | 0.564                   |
| 38              | 17.295                    | $\delta$ -Cadinene                                                               | 1.085                   |
| 39              | 17.318                    | (-)- $\alpha$ -Panasinsen                                                        | 0.499                   |
| 40              | 17.365                    | Cis- $\alpha$ -Bisabolene                                                        | 0.474                   |
| 41              | 17.407                    | 2,11-Epoxyacor-3-ene (Italicene - ether)                                         | 0.363                   |
| 42              | 17.458                    | $\alpha$ -Calacorene                                                             | 0.199                   |
| 43              | 17.481                    | 3,7,11-trimethyl-1,6,10-Dodecatrien-3-ol (E-farnesol)                            | 0.222                   |
| 44              | 17.532                    | (Z)-3,7-dimethyl-2,6-Octadien-1-ol acetate (Neryl acetate)                       | 0.461                   |
| 45              | 17.579                    | 2-Ethyl-1-pyrroline                                                              | 0.643                   |
| 46              | 17.621                    | 8-Trimethylsilyl-2,6-dimethylocta-2,5-dien-7-yne                                 | 2.533                   |
| 47              | 17.756                    | Caryophyllene oxide                                                              | 0.573                   |
| 48              | 17.798                    | Champacol (Guaiac alcohol)                                                       | 0.466                   |
| 49              | 17.867                    | 2-(2'0.3'0.4'-Trichlorophenoxy)-4,5,6-trichlorophenol                            | 0.144                   |
| 50              | 17.909                    | (1 S,6R)-3,7,7-Trimethylbicyclo[4.1.0]hept-3-en-2,5-dione                        | 1.437                   |
| 51              | 17.933                    | $\beta$ -Eudesmol                                                                | 0.412                   |
| 52              | 17.998                    | (S)-6-ethenyl-6-methyl-1-(1-methylethyl)-3-(1-ethylethylidene)-Cyclohexene       | 0.418                   |
| 53              | 18.044                    | $\beta$ -Selinol                                                                 | 0.657                   |
| 54              | 18.193                    | Veridiflorol                                                                     | 1.329                   |

**Table 4** Total phenolic contents (TPC) of film-forming gels formulations and *H. italicum* essential oil

| Film-forming gels formulations   | Total phenolic contents (mg GAE/g of the film-forming gels formulations) |
|----------------------------------|--------------------------------------------------------------------------|
| <i>H. italicum</i> essential oil | 124.60±0.20                                                              |
| F0                               | -                                                                        |
| F1                               | 9.25±0.025                                                               |
| F2                               | 17.35±0.055                                                              |
| F3                               | 35.60±0.115                                                              |

Values expressed are the mean ± standard deviation of three measurements. Total phenolic contents is expressed as gallic acid equivalent (GAE)

### *H. Italicum* Essential Oil and Film-forming Gel Formulations Enhance Cell Proliferation and Confers Antioxidant Protection

The inverted microscope images of the cells (Fig. 2A-C) revealed a marked increase in cell density following treatments, indicating enhanced cellular proliferation. Both *H. italicum* essential oil at 500 µg/mL (Fig. 2B) and the F2 film-forming gel formulation (Fig. 2C) promoted a visibly denser and more confluent cell monolayer compared to the control (Fig. 2A). The cell population in the F2-treated group (Fig. 2C) demonstrated a proliferative capacity, appearing significantly denser and more than both the control and the essential oil-treated groups. This observation suggests that the film-forming gel system more effectively stimulates fibroblast proliferation, a fundamental process for tissue regeneration and wound repair.

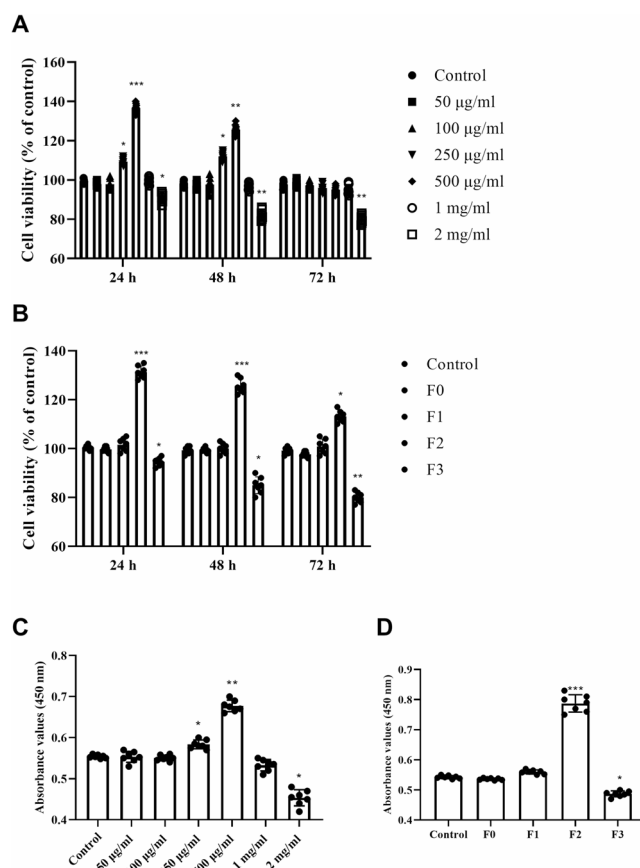
Given the pivotal role of oxidative stress in impairing dermal fibroblast functions relevant to wound healing, we next evaluated the antioxidant efficacy of the formulations. Analysis of intracellular superoxide levels using DHE revealed that both treatments significantly reduced oxidative stress. However, the F2 gel was markedly more effective. Quantitative analysis of fluorescence intensity (Fig. 2G) showed that while the essential oil (500 µg/mL) reduced ROS levels by 41%, the F2 formulation induced a more pronounced reduction of 65% compared to the control ( $n=7$ ,  $p<0.0001$ ). This antioxidant performance was visually evident in the corresponding fluorescence images (Fig. 2D-F).

To further characterize the antioxidant effects, we measured MDA and GSH levels, key markers of lipid peroxidation and cellular antioxidant capacity, respectively.

**Table 5** Effect of *H. italicum* essential oil and film-forming gel formulations containing this oil on collagenase enzyme

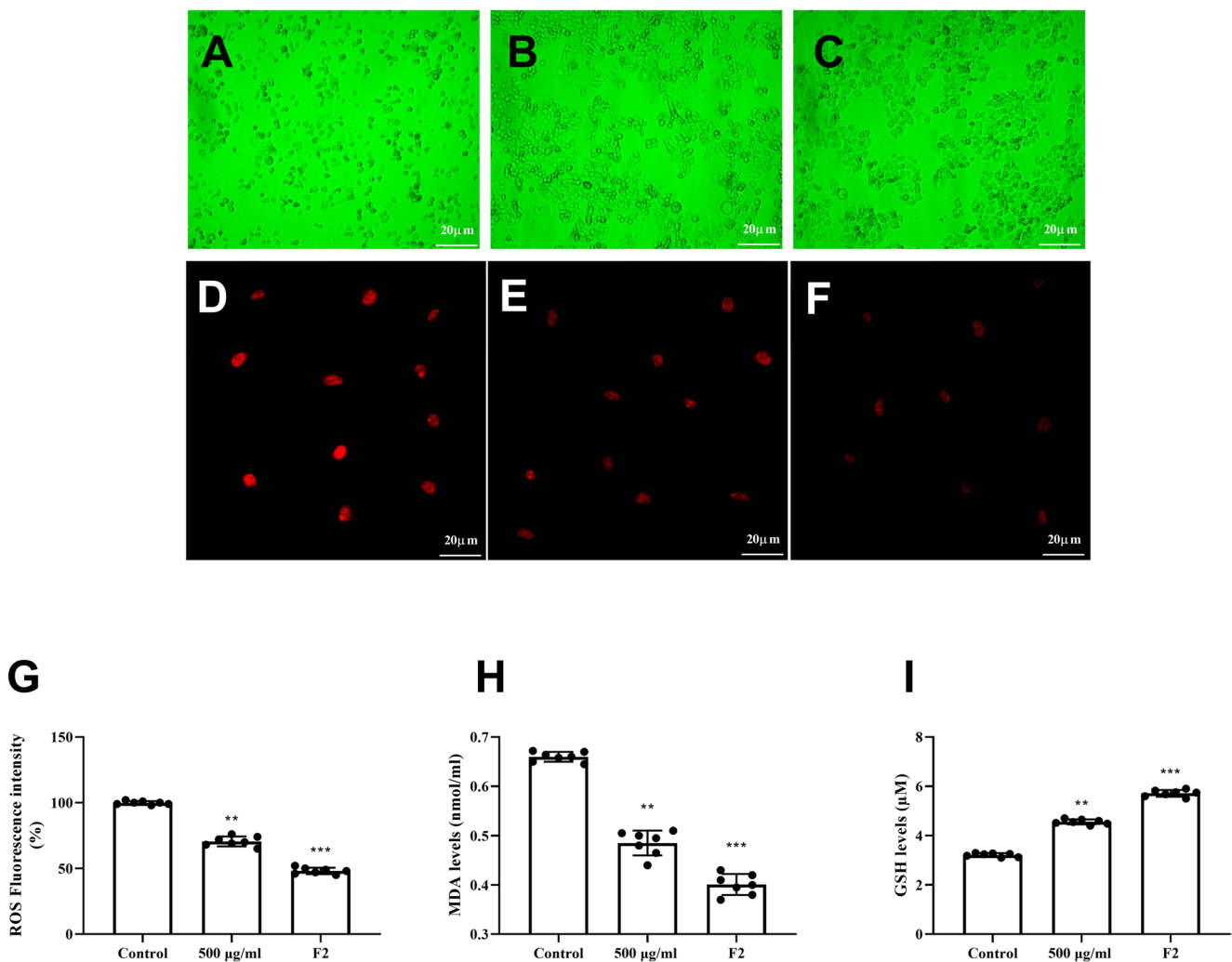
| Biological activity           | <i>H. italicum</i> essential oil IC <sub>50</sub> (µg/mL) | F0 IC <sub>50</sub> (µg/mL) | F1 IC <sub>50</sub> (µg/mL) | F2 IC <sub>50</sub> (µg/mL) | F3 IC <sub>50</sub> (µg/mL) |
|-------------------------------|-----------------------------------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Collagenase Type-I Inhibition | 35.73±0.47                                                | -                           | 429.81±0.74                 | 377.16±0.76                 | 237.13±0.76                 |

Values expressed are the mean ± standard deviation of three measurements



**Fig. 1** Cytotoxic and proliferative effects of *H. italicum* essential oil and film-forming gel formulations on dermal fibroblasts. **(A)** Cell viability assessed by CCK-8 assay after 24, 48, and 72 hours of treatment with *H. italicum* essential oil (50 µg/mL to 2 mg/mL). **(B)** Cell viability assessed by CCK-8 assay after 24, 48, and 72 hours of treatment with film-forming gel formulations (F0, F1, F2, F3). **(C)** Cell proliferation evaluated by BrdU assay after 24 hours of treatment with *H. italicum* essential oil. **(D)** Cell proliferation evaluated by BrdU assay after 24 hours of treatment with film-forming gel formulations. Data represent means ± SD of seven independent experiments ( $n=7$ ). \* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$

Consistent with the ROS data, the F2 formulation demonstrated a more protective effect against oxidative damage. As shown in Fig. 2H, the F2 gel reduced MDA levels by approximately 42%, a significantly greater reduction than the 35% decrease observed with the essential oil alone ( $n=7$ ,  $p=0.0004$ ). Concurrently, the F2 treatment increased intracellular GSH levels by 81%, substantially outperforming the essential oil, which raised GSH by 45% (Fig. 2I,



**Fig. 2** *H. italicum* essential oil and film-forming gel formulation enhance fibroblast proliferation and provide antioxidant protection. (A–C) Representative inverted microscope images showing cell density after 24-hour treatment: (A) Untreated control, (B) *H. italicum* essential oil (500  $\mu\text{g/ml}$ ), and (C) F2 film-forming gel formulation (0.5% w/w). (D–F) Intracellular superoxide levels detection using

DHE staining: (D) Control, (E) *H. italicum* essential oil (500  $\mu\text{g/ml}$ ), and (F) F2 formulation. (G) Quantitative analysis of ROS fluorescence intensity. (H) MDA levels as a marker of lipid peroxidation. (I) Intracellular GSH levels. Representative confocal microscopy images (20 $\times$  objective). Data represent means  $\pm$  SD of seven independent experiments ( $n=7$ ). Scale bar: 20  $\mu\text{m}$ . \*\* $p < 0.001$ , \*\*\* $p < 0.0001$

$n=7$ ,  $p=0.0001$ ). These findings suggest that the enhanced antioxidant response induced by F2 may directly contribute to improved fibroblast proliferation and migration, given the established inhibitory role of oxidative stress in wound repair.

### *H. italicum* Formulations Modulate Key Extracellular Matrix Components in Dermal Fibroblasts

Immunocytochemical analysis was employed to evaluate the effect of *H. italicum* treatments on the expression of ECM proteins: COL1A1, FN1, and MMP7. Representative fluorescence microscopy images (Fig. 3A) revealed a substantial increase in the immunofluorescence intensity for

all three proteins in treated groups compared to the control. Qualitative assessment indicated that the F2 film-forming gel formulation induced a more pronounced expression of COL1A1, FN1, and MMP7 than the essential oil (500  $\mu\text{g/ml}$ ) alone.

Quantitative analysis of fluorescence intensity corroborated these observations. As shown in Fig. 3B, the F2 formulation significantly increased COL1A1 levels by  $85.2 \pm 4.7\%$ , a markedly greater enhancement than the  $55\%$  increase elicited by the essential oil ( $n=7$ ,  $p < 0.0001$ ). Similarly, the expression of FN1 was more effectively stimulated by the F2 gel, which elevated its levels by  $74.8 \pm 5.1\%$  compared to a  $40.6 \pm 3.8\%$  increase with the essential oil (Fig. 3C,  $n=7$ ,  $p < 0.0001$ ). The expression profile of MMP7, a

protease involved in ECM remodeling, also demonstrated a similar trend. The F2 treatment upregulated MMP7 by  $60.3 \pm 4.9\%$ , outperforming the essential oil, which caused a 28% increase (Fig. 3D,  $n=7$   $p=0.0002$ ). MMP7 is traditionally recognized for its proteolytic activity against ECM components including fibronectin and collagen IV. However, controlled MMP activity is essential for proper ECM remodeling, as excessive inhibition leads to fibrosis and disorganized matrix deposition. Therefore, the observed upregulation of MMP7 by F2 formulation ( $60.3 \pm 4.9\%$  increase) may be interpreted as a balanced pro-remodeling response rather than unopposed degradation, particularly given the concurrent increases in COL1A1 ( $85.2 \pm 4.7\%$ ) and FN1 ( $74.8 \pm 5.1\%$ ). The concurrent increase in COL1A1 and FN1 alongside MMP7 supports a coordinated ECM remodeling response rather than excessive matrix degradation.

### ***H. Italicum* Formulations Accelerate Fibroblast Migration in a Scratch Wound Assay**

The impact of *H. italicum* formulations on the migratory capacity of dermal fibroblasts, a critical event in wound re-epithelialization, was evaluated using a scratch wound assay. Representative micrographs captured at 0 and 24 h post-scratch (Fig. 4A) visually demonstrated enhanced wound closure in both treatment groups compared to the control. Qualitatively, the F2 film-forming gel formulation facilitated a more rapid and complete repopulation of the scratch area over the 24-hour period compared to the essential oil (500  $\mu\text{g/mL}$ ) alone.

Quantitative image analysis (Fig. 4B) confirmed these observations. After 24 h, treatment with *H. italicum* essential oil significantly promoted wound closure, resulting in  $52.3 \pm 4.1\%$  healing of the initial scratch area. In contrast, the F2 film-forming gel exhibited a superior pro-migratory effect, achieving nearly  $84.7 \pm 5.3\%$  wound closure within the same timeframe, a statistically significant enhancement over both the control and the essential oil treatment ( $p < 0.0001$ ). The control group showed only about  $24.8 \pm 3.2\%$  closure under the same conditions.

### ***H. Italicum* Formulations Upregulate ECM-related Gene and Protein Expression**

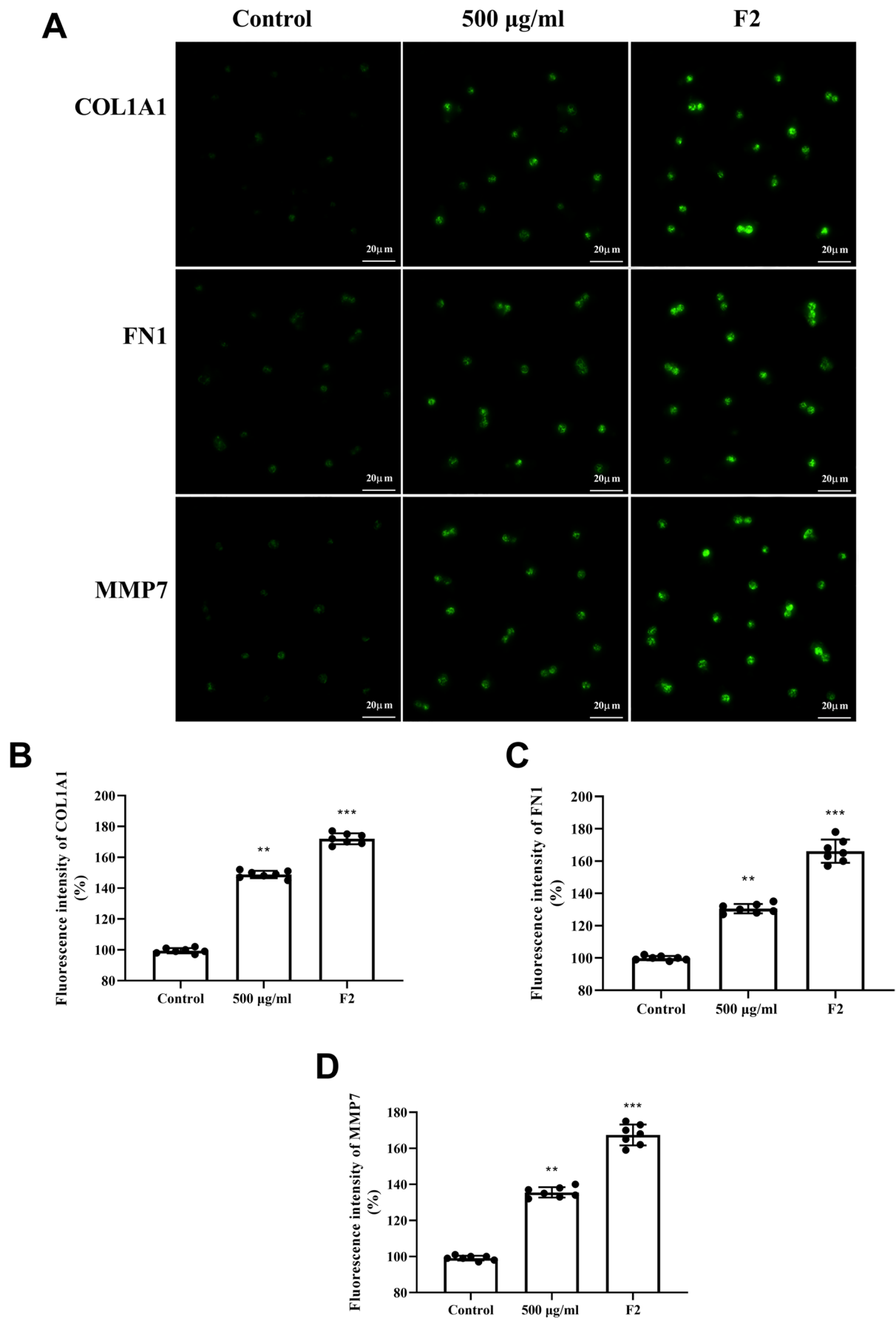
To further elucidate the molecular mechanisms underlying the enhanced dermal fibroblast functions relevant to wound healing properties, we analyzed the expression of key ECM components at both the protein and transcriptional levels. Western blot analysis (Fig. 5A) confirmed that treatment with *H. italicum* essential oil (500  $\mu\text{g/mL}$ ) and the F2 film-forming gel significantly increased the protein levels of COL1A1, FN1, and MMP7 compared

to the control. Densitometric quantification revealed that the F2 formulation induced a more substantial upregulation than the essential oil alone. Specifically, F2 formulation increased COL1A1 protein levels to  $1.51 \pm 0.12$ -fold, FN1 to  $1.44 \pm 0.09$ -fold, and MMP7 to  $1.67 \pm 0.14$ -fold relative to control ( $p < 0.0001$ ). In contrast, the essential oil treatment resulted in increases of 32%, 28%, and 38% for the same proteins.

Consistent with the protein data, qRT-PCR analysis demonstrated a parallel upregulation at the mRNA level (Fig. 5B-D). qRT-PCR analysis revealed that F2 formulation significantly upregulated COL1A1 mRNA expression by  $2.67 \pm 0.31$ -fold ( $n=7$ ,  $p=0.0004$ ), FN1 by  $3.85 \pm 0.42$ -fold ( $n=7$ ,  $p=0.0001$ ), and MMP7 by  $2.72 \pm 0.28$ -fold ( $n=7$ ,  $p=0.0003$ ) relative to untreated control. The essential oil (500  $\mu\text{g/mL}$ ) also significantly increased COL1A1 ( $1.41 \pm 0.19$ -fold,  $n=7$ ,  $p=0.012$ ), FN1 ( $1.55 \pm 0.22$ -fold,  $n=7$ ,  $p=0.008$ ), and MMP7 ( $1.48 \pm 0.17$ -fold,  $n=7$ ,  $p=0.015$ ). The parallel increases observed at both mRNA and protein levels strengthen the mechanistic link between transcriptional activation and functional ECM remodeling capacity.

## **Discussion**

The management of impaired wound healing remains a significant clinical challenge, driving the continual search for innovative therapeutic strategies that can effectively modulate the complex cellular and molecular processes of tissue repair. In this context, plant-derived compounds have garnered considerable attention due to their multi-target biological activities, biocompatibility, and historical efficacy in traditional medicine. To evaluate the therapeutic potential of our novel formulations within this landscape, we first sought to contextualize their bioactivity against established benchmarks reported in the literature. For instance, dexpantenol, a standard wound-healing promoter, has been shown to activate fibroblast proliferation both in vitro and in vivo [19]. Similarly, allantoin is reported to significantly enhance cell migration in scratch assays; for example, studies have demonstrated allantoin-induced wound closure rates of up to 94% over 72 h [20]. In comparison, our F2 formulation achieved a 40.7% increase in proliferation (BrdU assay) and 84.7% wound closure within 24 h, which is comparable to or exceeds these benchmarks. Furthermore, the 2.67-fold upregulation of COL1A1 mRNA by F2 aligns with the effects of TGF- $\beta$ 1, a potent pro-fibrotic growth factor, which has been shown to robustly induce collagen type I expression in fibroblasts [21]. To date, no study has systematically compared the wound-healing efficacy of *H. italicum* essential oil in its free form with that of a novel film-forming gel



**Fig. 3** *H. italicum* formulations upregulate ECM protein expression in dermal fibroblasts. **(A)** Representative immunofluorescence images showing COL1A1, FN1, and MMP7 expression after 24-hour treatment with untreated control, *H. italicum* essential oil (500 µg/mL), and F2 film-forming gel formulation. **(B–D)** Quantitative analysis of fluorescence intensity for **(B)** COL1A1, **(C)** FN1, and **(D)** MMP7. Representative confocal microscopy images (40× oil immersion objective). Data represent means ± SD of seven independent experiments (n=7). Scale bar: 20 µm. \*\*p < 0.001, \*\*\*p < 0.0001

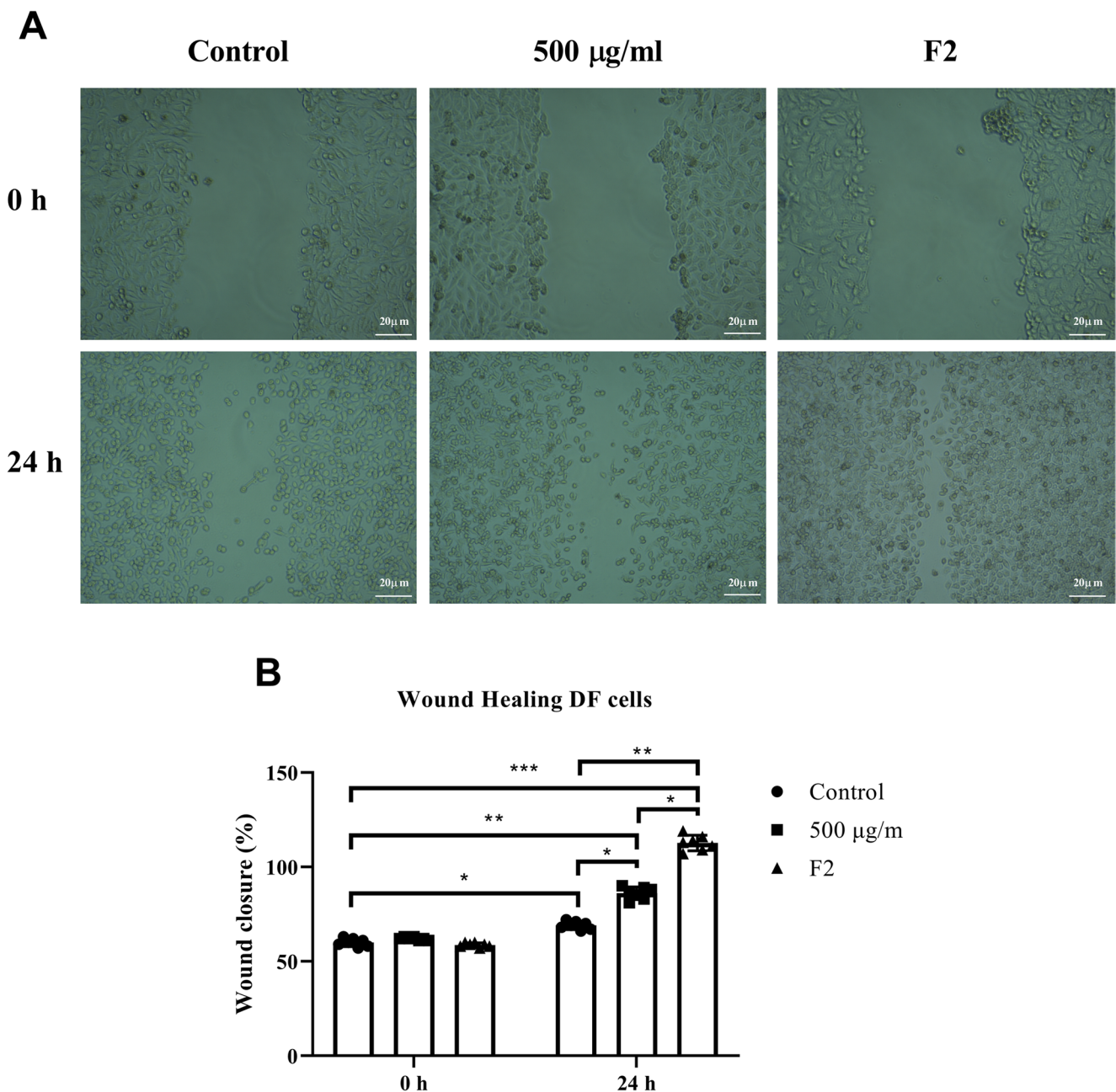
formulation containing the oil in an in vitro human dermal fibroblast model. This study represents the first comprehensive investigation to integrate phytochemical characterization, antioxidant and anticollagenase assessments, cellular bioactivity profiling, and molecular analyses to elucidate the wound-healing potential of *H. italicum* within an advanced topical delivery system.

The physicochemical characteristics of the developed film-forming gel formulations were consistent with the requirements for dermal applications. All formulations exhibited suitable viscosity, pH, and homogeneity, indicating their potential for skin compatibility and ease of application. The pH values were within the normal skin range, minimizing the risk of irritation [22]. Polyvinyl alcohol (PVA) was chosen as the primary polymer due to its chemical stability, biocompatibility, and film-forming properties, which are well-documented in the literature [23, 24]. Blending PVA with carboxymethyl cellulose (CMC) improved elasticity and hydrophilicity, in agreement with previous studies showing that PVA–CMC mixtures enhance film flexibility and adhesion [25]. These features are critical for maintaining a protective barrier over the wound while providing a moist environment conducive to healing, a key aspect of modern wound dressings.

The GC–MS profile of the essential oil was in line with earlier reports describing  $\gamma$ -curcumene,  $\alpha$ -pinene,  $\beta$ -selinene, and neryl acetate as major constituents of *H. italicum* essential oil [26, 27]. It is well-documented that the relative abundance of these key components exhibits intraspecific variation, influenced by environmental factors such as geographical origin, phenological stage, soil properties, altitude, climatic conditions, and the extraction methodology employed [28, 29]. Notably, two major components, neryl acetate and pinene, have been directly implicated in the wound healing process. Neryl acetate contributes to the restoration of the skin barrier by upregulating enzymes involved in the glucosylceramide pathway, thereby increasing the lipid and ceramide content of the stratum corneum. Conversely, pinene promotes collagen deposition, leading to the formation of scars with improved tensile strength. Together, these compounds facilitate epidermal repair by enhancing keratinocyte differentiation, intercellular cohesion, and the development of the cornified envelope [30].

The essential oil demonstrated a high phenolic content compared to the gel formulations, consistent with previous studies on *Helichrysum* species. For instance, methanol extracts of 16 Turkish *Helichrysum* species were shown to contain 66.74 to 160.63 mg GAE/g of dry extract, a range that aligns with the value obtained for the essential oil in this study [31]. A subsequent investigation by Albayrak et al. further confirmed this variation, reporting TPC values of  $71.81 \pm 1.0$  to  $144.50 \pm 1.2$  mg GAE/g extract across four different species [32]. Further illustrating the variability influenced by plant part, a study on *H. italicum* subsp. reported the highest TPC in the whole plant ( $271.36 \pm 5.3$  mg GAE/g), followed by the flowers ( $221.04 \pm 6.7$  mg GAE/g) and leaves ( $153.12 \pm 4.6$  mg GAE/g) [33]. The disparities in TPC observed across these studies can likely be attributed to several factors, including the plant's collection time, geographical location, seasonal variations, the specific plant part used, and the extraction methodology [34].

Collagenase, a matrix metalloproteinase, is the enzyme that catalyzes the breakdown of collagen, the main protein of the skin. Collagen production, one of the most important steps of wound healing processes, can be negatively affected by reactive oxygen species formed as a result of external factors (excessive exposure to sunlight) and increase the activity of the collagenase enzyme, which can delay the wound healing process [35]. Therefore, inhibition of collagenase enzyme is gaining importance. Recent investigations have established that bioactive compounds from natural products, including essential oils and plant extracts, can function as collagenase inhibitors [36]. Supporting this, *H. italicum* essential oil of Italian origin, characterized by high concentrations of neryl acetate (15.75%),  $\alpha$ -pinene (8.21%), and limonene (4.55%), has been shown to exhibit in vitro anti-collagenase and anti-elastase activity [37]. Further evidence comes from a study on *H. italicum* extracts obtained via a green ultrasound-assisted method, which demonstrated anti-collagenase activity with IC<sub>50</sub> values of  $12.035 \pm 1.029$  and  $14.392 \pm 0.705$  µL extract/mL, respectively [38]. The anti-collagenase activity of *H. italicum* essential oil is proposed to be primarily mediated by limonene and  $\alpha$ -pinene. This is corroborated by separate findings; Chidambara et al. reported that d-limonene-rich blood orange essential oil reduced MMP-9 expression [39], while Rufino et al. documented the inhibitory effect of  $\alpha$ -pinene on collagenase (specifically MMP-1 and MMP-13) expression [40]. In this study, both the essential oil and the gel formulations exhibited inhibitory activity against collagenase type I, with the essential oil showing the strongest effect. The retention of activity within the gels suggests that the film-forming system preserves and gradually releases active compounds while maintaining their enzymatic inhibitory potential.



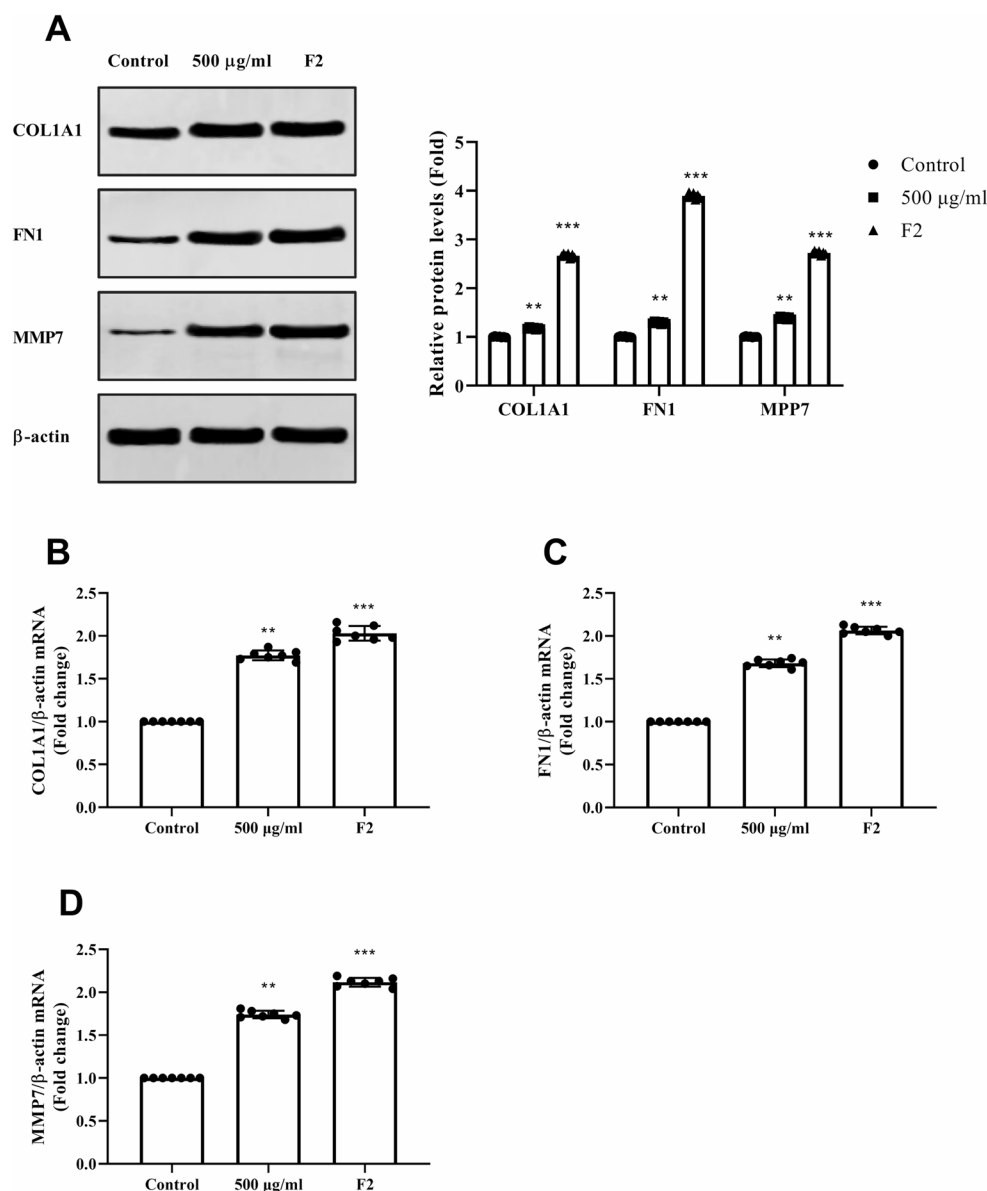
**Fig. 4** *H. italicum* formulations promote fibroblast migration in a scratch wound assay. **(A)** Representative phase-contrast images of scratch wounds at 0 and 24 hours after treatment with untreated control, *H. italicum* essential oil (500  $\mu\text{g/mL}$ ), and F2 film-forming gel formulation. **(B)** Quantitative analysis of wound closure at 24 h, calcu-

lated as  $[(\text{Area}_{0\text{h}} - \text{Area}_{24\text{h}}) / \text{Area}_{0\text{h}}] \times 100$ . Representative phase-contrast microscopy images (40 $\times$  objective). Data represent means  $\pm$  SD of seven independent experiments ( $n=7$ ). Scale bar: 20  $\mu\text{m}$ . \* $p < 0.05$ , \*\* $p < 0.001$ , \*\*\* $p < 0.0001$

The search for effective wound healing agents has increasingly turned towards natural products like *H. italicum*, valued for their multi-faceted therapeutic activities and favorable safety profiles [9]. Concurrently, advances in pharmaceutical technology have highlighted the benefits of film-forming systems, which offer improved residence time and sustained drug release compared to conventional formulations [12]. This study aimed to leverage both these

advantages by directly comparing the efficacy of *H. italicum* essential oil with a novel *H. italicum*-loaded film-forming gel in an in vitro wound healing model. These findings align with previous reports demonstrating that bioactive constituents of *H. italicum*, including  $\alpha$ -pinene and caffeoylquinic acid derivatives, stimulate fibroblast proliferation and viability while excessive doses may exert cytotoxic effects due to oxidative imbalance [11]. Comparable results have been

**Fig. 5** *H. italicum* formulations upregulate ECM-related gene and protein expression in dermal fibroblasts. **(A)** Western blot analysis and relative quantification of COL1A1, FN1, and MMP7 protein levels after treatment with *H. italicum* essential oil (500 µg/mL) and F2 film-forming gel formulation. **(B–D)** qRT-PCR analysis of **(B)**COL1A1, **(C)**FN1, and **(D)**MMP7 mRNA expression levels. Data represent means  $\pm$  SD of seven independent experiments ( $n=7$ ). \*\* $p < 0.001$ , \*\*\* $p < 0.0001$



observed with other natural biopolymers such as sericin, which enhances fibroblast proliferation and collagen synthesis without cytotoxicity, thereby facilitating wound healing [41]. A time-dependent decline in viability was observed across all groups, including the untreated control, including untreated control, between 24 h and 72 h (Fig. 1A–B). This is likely attributable to nutrient depletion and contact inhibition as cells reached confluency, rather than specific treatment toxicity. Notably, the F2 formulation maintained significantly higher viability than untreated control at all time points ( $p < 0.05$ ), indicating sustained cytoprotective activity even under suboptimal culture conditions. These results demonstrate that both *H. italicum* essential oil and its incorporation into a film-forming gel can significantly support and enhance key cellular processes critical for dermal fibroblast functions relevant to wound healing. The essential

oil at intermediate concentrations (250–500 µg/mL) and the F2 gel formulation (0.5% w/w) consistently promoted both fibroblast viability and proliferation. Based on this optimal balance of bioactivity and excellent biocompatibility, the 500 µg/mL essential oil and F2 formulations were selected for subsequent investigations into their dermal fibroblast functions relevant to wound healing mechanisms. The observed bioactivities can be attributed to the major constituents identified.  $\alpha$ -Pinene (13.866%), previously shown to stimulate collagen deposition and fibroblast proliferation [30], likely contributes to the pro-migratory and ECM-promoting effects. Neryl acetate (8.266%) has been reported to enhance skin barrier function via upregulation of ceramide synthesis enzymes [30].  $\gamma$ -Curcumen (15.563%) and  $\beta$ -selinene (8.444%) are sesquiterpenes with documented antioxidant and anti-inflammatory properties [26, 27]. The

presence of D-limonene (4.307%) may contribute to the observed anti-collagenase activity, as this compound has been shown to inhibit MMP expression [39].

Oxidative stress is a pivotal factor that impairs wound healing, particularly in chronic wounds such as diabetic ulcers [8]. Our antioxidant results are consistent with previous evidence showing that *H. italicum* essential oil and hydrolates mitigate oxidative stress through their phenolic and terpene content [11]. Since excessive ROS delays wound healing by impairing fibroblast migration and ECM synthesis [42], the ability of F2 to strongly attenuate ROS while increasing GSH positions it as a superior therapeutic candidate. Similar findings have been reported in diabetic wound models, where *H. italicum* ointments improved redox balance and enhanced healing outcomes [14]. While some reports indicate a relatively modest in vitro antioxidant capacity for *H. italicum* essential oil alone, often due to solubility issues in polar assays [43], its incorporation into a topical formulation appears to enhance its in situ efficacy, as demonstrated by our results and other in vivo studies [14]. These findings conclusively show that the novel film-forming gel (F2) not only promotes fibroblast proliferation but also provides a significantly enhanced antioxidant benefit over the essential oil alone. It more effectively scavenges ROS, mitigates lipid peroxidation, and boosts the endogenous antioxidant defense system, thereby creating a more conducive microenvironment for the fibroblast function and survival necessary for efficient dermal fibroblast functions relevant to wound healing.

The upregulation of COL1A1 and FN1 is a strong indicator of enhanced collagen deposition and tissue strength, which are vital for effective wound closure and scar integrity [11]. The upregulation of COL1A1 mRNA and protein suggests enhanced collagen synthesis capacity, which is consistent with previous in vivo studies reporting increased hydroxyproline content following treatment with *H. italicum* formulations [44]. However, hydroxyproline was not directly measured in the present study and should be assessed in future histological analyses. The parallel increase in MMP7, a protease involved in ECM turnover, suggests a balanced promotion of both synthesis and remodeling, preventing the formation of a disorganized ECM. Our results are supported by previous work indicating that *H. italicum* essential oil can influence tissue remodeling-related proteins [12]. The finding that  $\alpha$ -pinene, a major component of the oil, contributes to collagen deposition provides a phytochemical basis for our observations. These findings demonstrate that both *H. italicum* essential oil and the F2 film-forming gel promote the synthesis of key ECM proteins critical for tissue repair. The concurrent upregulation of COL1A1, FN1, and MMP7 by the F2 formulation suggests a coordinated promotion of both ECM synthesis and remodeling. While MMP7 is

capable of degrading several ECM components, its role in wound healing is context-dependent and not inherently detrimental. Controlled proteolytic activity is essential for removing damaged matrix, facilitating cell migration, and releasing matrix-bound growth factors [45]. Moreover, MMP7 has been shown to cleave E-cadherin, thereby promoting keratinocyte migration and re-epithelialization [46]. Therefore, the observed 60% increase in MMP7 expression, when accompanied by 85% and 75% increases in COL1A1 and FN1 respectively, is interpreted as a pro-remodeling rather than a pro-degradative signal. Nevertheless, direct measurement of MMP7 enzymatic activity and assessment of TIMP expression will be necessary to definitively establish the net proteolytic balance. While our study prioritized the initial feasibility, biological performance, and cellular mechanisms of the novel *H. italicum* FFG, we acknowledge that a comprehensive physical characterization—including detailed analysis of drying time, adhesion, mechanical strength, and drug release kinetics—was beyond the scope of this work. These critical parameters will be the focus of dedicated formulation optimization and physical characterization studies in the near future. However, the film-forming gel system is significantly more efficacious, markedly enhancing the production of the structural proteins COL1A1 and FN1, as well as the remodeling enzyme MMP7. This performance suggests that the film-forming gel not only delivers the active compounds more effectively but also creates a microenvironment that optimally supports fibroblast-mediated ECM synthesis and reorganization, accelerating the progression of the proliferative and remodeling phases of dermal fibroblast functions relevant to wound healing.

The film formed in situ may also maintain a hydrated environment and protect the wound bed, further facilitating cell movement. This aligns with observations from other studies, where *H. italicum* hydrolate was shown to accelerate cell migration and improve wound closure, an effect attributed to its phenolic compounds like chlorogenic acid and naringenin [47]. Previous in vivo studies have confirmed that *H. italicum*-based gels and ointments significantly improve wound contraction, collagen content, and angiogenesis [11]. Moreover, the superior performance of gels compared to ointments may be attributed to their rapid coagulative and easy-to-apply nature [12]. These results quantitatively demonstrate that the F2 film-forming gel formulation significantly augments the migratory potential of dermal fibroblasts, outperforming the essential oil by a considerable margin. This enhanced cell movement, combined with the previously observed proliferation, indicates that the film-forming gel system more effectively stimulates the key cellular behaviors required for efficient wound contraction and closure.

The coordinated upregulation of COL1A1, FN1, and MMP7 at both mRNA and protein levels by the F2 formulation provides strong evidence of its role in stimulating fibroblast-driven ECM remodeling. These findings are consistent with previous reports showing that *H. italicum* constituents regulate ECM gene expression and remodeling enzymes, thereby accelerating wound repair [14]. Comparable molecular effects have been documented with hydrolates rich in phenolic compounds such as naringenin and chlorogenic acid, which regulate collagen secretion and angiogenesis [11]. These results demonstrate that the *H. italicum*-loaded film-forming gel (F2) upregulates the expression of critical ECM components, not only by enhancing their protein synthesis but also by activating their transcription. The superior efficacy of the F2 formulation in modulating both gene and protein expression of COL1A1, FN1, and MMP7 provides a molecular basis for its enhanced performance in promoting ECM synthesis and remodeling, further validating its potential as a highly effective topical system for accelerating wound repair. The superior performance of F2 formulation compared to free essential oil likely arises from several interconnected mechanisms. First, the film-forming gel provides a hydrated microenvironment that maintains fibroblast viability and supports cell migration, consistent with the known benefits of moisture-retentive dressings [48]. Second, the polymeric matrix (PVA/CMC) may stabilize labile essential oil components, particularly oxygenated terpenes, by reducing volatilization and oxidative degradation [49]. Third, upon application, the gel undergoes solvent evaporation and forms a thin film, which concentrates the active compounds at the application site and prolongs their residence time, thereby enhancing local bioavailability [7]. Fourth, the gradual diffusion of essential oil from the swollen polymer network may provide sustained exposure of fibroblasts to sub-cytotoxic concentrations, avoiding the peak-and-trough profile associated with bolus application of free oil. This interpretation is supported by the observation that F2 (0.5% w/w) outperformed both lower (F1) and higher (F3) concentrations, indicating an optimal balance between efficacy and toxicity that is likely facilitated by controlled release. Upon application to the aqueous culture medium, the components of the liquid gel formulation disperse, facilitating the delivery of the essential oil compounds to the cells. This approach mimics the clinical application of the gel, which is designed to be applied as a liquid that subsequently forms a protective film in situ on the wound. While our study prioritized the initial feasibility, biological performance, and cellular mechanisms of the novel *H. italicum* FFG, we acknowledge that a comprehensive physical characterization—including detailed analysis of drying time, adhesion, mechanical strength, and drug release kinetics—was beyond the scope of this work. These critical parameters will be the focus of dedicated formulation optimization and physical characterization studies in the near future.

## Conclusion

In conclusion, our integrated results and discussion demonstrate that the novel film-forming gel formulation (F2) of *H. italicum* essential oil offers significant advantages over the essential oil alone. It provides a superior platform for enhancing fibroblast viability, proliferation, and migration; confers antioxidant protection; and potentially stimulates the synthesis and remodeling of key extracellular matrix components through the upregulation of associated genes and proteins. The mechanism appears to involve the creation of a protective microenvironment by the polymeric film, which likely enhances local bioavailability and prolongs fibroblast exposure to sub-cytotoxic concentrations of the active compounds. While formal release kinetics studies are necessary to confirm this interpretation, the enhanced bioactivity observed with the F2 formulation strongly supports this hypothesis. These findings support the hypothesis that incorporating *H. italicum* into an advanced film-forming delivery system can significantly augment its wound healing potential, positioning it as a candidate worthy of further preclinical evaluation in appropriate in vivo models. However, it is important to acknowledge the limitations of this study. First, all experiments were conducted using a monoculture of dermal fibroblasts, which does not recapitulate the complex multicellular environment of wound healing involving keratinocytes, endothelial cells, macrophages, and other immune cells. The absence of endothelial cells precludes assessment of angiogenic effects, while the lack of keratinocytes limits conclusions regarding re-epithelialization. Second, while the F2 formulation demonstrated superior efficacy in vitro, these findings require validation in appropriate in vivo models. Future studies should evaluate wound closure rates, histological parameters, and biomarker expression in full-thickness excisional wound models, preferably in diabetic or aged animals to model impaired healing conditions. Third, the film-forming gel formulation was tested using a single polymer composition (PVA/CMC). Systematic optimization of polymer ratios, plasticizer content, and essential oil concentration could further improve performance. Additionally, we did not conduct formal stability studies (accelerated or long-term), nor did we evaluate skin irritation potential using reconstructed human epidermis (RHE) models or in vivo patch testing. Fourth, although MMP7 upregulation was observed at both mRNA and protein levels, we did not measure actual MMP7 enzymatic activity. Zymography or FRET-based activity assays would be required to confirm whether the increased MMP7 protein corresponds to increased proteolytic activity or reflects a compensatory mechanism. Similarly, the expression of tissue inhibitors of metalloproteinases

(TIMPs) was not assessed; the net MMP/TIMP balance is critical for determining ECM remodeling outcomes. Fifth, the claim of “sustained release” is inferential based on enhanced bioactivity, but no formal drug release kinetics studies were performed. Future work should include Franz cell diffusion studies to quantify essential oil release profiles from the film-forming gel. Finally, this study focused on a single *H. italicum* essential oil chemotype. Given the known variability in essential oil composition based on geographical origin and harvest time, generalizability to other chemotypes should be established. Despite these limitations, the robust and consistent enhancement of fibroblast function by the F2 formulation provides a strong rationale for advancing this technology toward preclinical development.

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**Data Availability** The raw data supporting the findings of this study are available from the corresponding author upon reasonable request. Supplementary figures (Uncropped Western blot images, GC-MS chromatogram, standard curves, inhibition curves) are provided in the online supplementary material.

## Declarations

**Competing interest** The authors declare no competing interests.

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