

# The *in-vitro* Wound Healing Potential of Essential Oil Extracted from *Mentha longifolia* L.

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**ABSTRACT:** Products from *Mentha* species are widely used in medical applications, including wound healing, due to the bioactive compounds they may contain. However, data on the effect of *Mentha longifolia* L. on wound healing are limited. This study investigated the antimicrobial and wound healing-promoting effects of *Mentha longifolia* L. essential oil using *in vitro* methods. The chemical compositions of the essential oil were identified using gas chromatography/mass spectrometry, while the agar well diffusion and disk diffusion methods were used to determine antimicrobial activity against pathogenic strains. A scratch wound healing assay was performed following determination of the cytotoxic dose in human fibroblast cell lines. The media were used for biochemical analysis 48 h after the *in vitro* wound model. Immunohistochemical staining was applied to determine the contribution of the essential oil to wound healing. The essential oil exhibited varying levels of antimicrobial activity against the tested pathogens and increased cell viability. All doses in the scratch wound healing assay promoted wound closure in a shorter time than in the control group. TAC levels were higher in the treated groups than in the control group, while TOS, IL-6, and TNF- $\alpha$  levels were lower. Levels of expression of FGF 2, IGF, and TGF- $\beta$  were higher in the treated groups than in the control group at increasing doses. The essential oil of *Mentha longifolia* L. exhibited antimicrobial effects and improved wound healing at doses of 5  $\mu$ g/mL and 10  $\mu$ g/mL.

**KEYWORDS:** *Mentha longifolia* L.; wound healing; fibroblast; essential oil; antimicrobial effect

## 1. INTRODUCTION

Wounds disrupt the body's normal integrity due to external agency action. Acute wounds, which heal in approximately six weeks, occur after various traumas or surgical procedures. However, this period may last for up to three months, depending on the patient's age and physiological condition, and the presence of chronic diseases or postoperative infections. Wounds that have not healed in more than three months are now defined as chronic, and the therapeutic approach to these is changing [1]. The most common chronic wounds in elderly and/or bedridden patients are pressure sores, venous leg ulcers, and diabetic foot ulcers. Millions of people worldwide are affected by acute and chronic wounds that develop for various reasons every year. Delayed or unsuccessful wound treatment may cause bacterial infections, prolonged hospital stays, surgical interventions, loss of limbs, or death. This process also results in increased health costs [2].

The wound healing process involves in four states: hemostasis, inflammation, proliferation, and remodeling phases [3]. Inadequate wound care often results in the formation of infected wounds. Oxidative stress, primarily caused by chemical mediators released during inflammation, delays wound healing. Free radicals, a byproduct of metabolism and highly unstable molecules, provide a line of defense against invading

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organisms. However, overproduction of these radicals causes damage through chemical reactions with nucleophilic tissue components, such as nucleic acids, proteins, and polyunsaturated fatty acids in cell and organelle membranes [1]. Antioxidant and anti-inflammatory compounds are therefore critical agents that accelerate the healing process through inflammation and proliferation phases. Plants are a natural source of antioxidant and anti-inflammatory compounds [4,5]. Although several strategies are available for obtaining these secondary metabolites, essential oils are the most common and the oldest method for extracting these components [6]. Aromatic plants produce extraordinary compounds in the form of secondary metabolites. The essential oil combines hydrocarbons, alcohols, aldehydes, esters, ethers, ketones, oxides, phenols, and terpenes with a characteristic smell [7].

*Mentha longifolia* L., a perennial herb the *Lamiaceae* family, also known as wild mint or Asian mint, is widely grown in Mediterranean countries, Europe, Australia, and North Africa. The *Lamiaceae* family includes 236 genera and more than 7,000 species and represents a significant proportion of medicinal and aromatic plants [8]. *Mentha* species, known for their pungent aroma, are used in traditional medicine to treat various diseases and for symptom relief [9]. *Mentha longifolia* L. essential oil has been used to treat menstrual irregularities, pulmonary infections, colds, shortness of breath, allergic asthma, kidney stones and urinary tract infections, abdominal pain, indigestion, gastrointestinal complaints, backache, headache, and wounds [9,10]. Experimental studies have confirmed that some species of mint are effective in wound healing. A previous study reported faster healing among rats with second-degree burn wounds in a group treated with *Mentha pulegium* methanol extract compared to a control group [11]. Researchers modeling infected wounds in mice also observed decreased inflammation and more rapid wound following topical application of *Mentha piperita* essential oil [12].

Although *Mentha longifolia* L. is widely distributed worldwide, studies of its effect on wound healing are limited. This study investigated the antimicrobial activity of *Mentha longifolia* L. essential oil and its effect on wound healing in the human dermal fibroblast cell line.

## 2. RESULTS AND DISCUSSION

### 2.1. GC/MS analysis

Following the *Mentha longifolia* L. essential oil (MLEO) GC/MS analysis, 26 components were identified, constituting 76.39% of the essential oil. The main components of this light-yellow essential oil, mainly consisting of oxygenated monoterpenes, were piperitone, piperitenone oxide, piperitenone 1,8-cineole, and caryophyllene oxide (Figure 1 and Table 1). Examination of the MLEO components obtained from different parts of the world revealed that the findings of the present research were compatible with those in the previous literature [6,13,14].

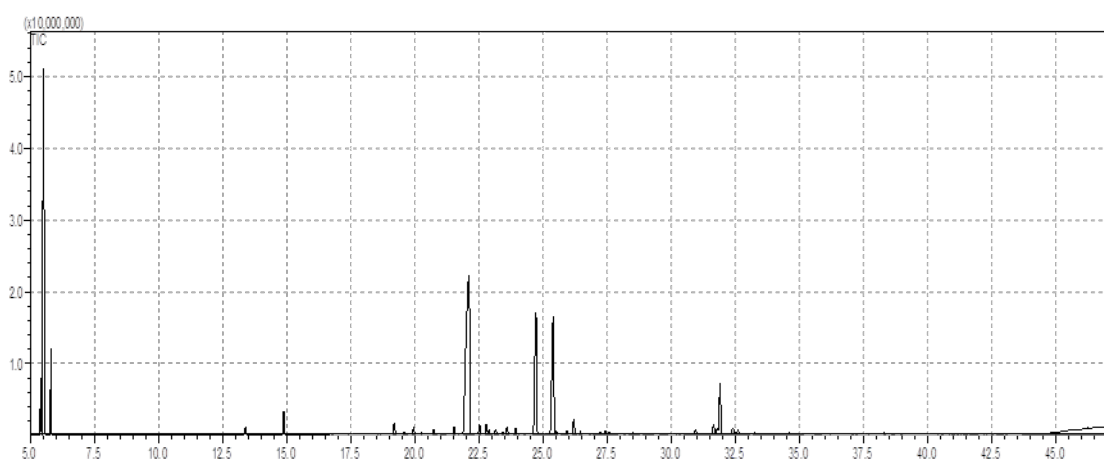


Figure 1. Chromatogram of Sample MLEO

**Table 1.** Results of sample MLEO GC/MS

	Retention Time	Area	% Area	Height	Name	Similarity Rate, %
1	5.402	16853308	2,21	7915910	3-Methylpentane	98
2	5.800	24304502	3,18	11993738	Methylcyclopentane	96
3	13.390	2660042	0,35	1002472	Ethyl amyl carbinol	96
4	14.570	220221	0,13	85497	p-Cymene	96
5	14.738	366242	0,25	133548	Limonene	96
6	14.879	9795326	4,28	3367200	1,8-Cineole	95
7	15.870	204850	0,03	80024	Hexanoic acid	92
8	16.781	786472	1,1	252175	Linalool	96
9	19.582	1063615	0,84	337343	4-Terpineol	92
10	19.690	207184	0,03	59530	p-Cymen-Alpha-Ol	92
11	19.946	3999160	1,52	1122650	$\alpha$ -Terpineol	96
12	20.252	892883	0,12	288075	Myrtenal	95
13	21.036	395350	0,05	146083	Cis-3-Hexenyl valerate	96
14	21.532	3715697	1,49	1139115	Pulegone	95
15	21.717	262936	0,03	86749	Linalyl acetate	91
16	22.105	2,16E+08	28,32	22257427	Piperitone	97
17	22.782	5666392	3,74	1371583	Thymol	96
18	23.146	2650667	0,35	622119	Carvacrol	91
19	24.045	459997	0,06	146809	Myrtenyl acetate	93
20	24.721	83838827	10,98	16913524	Piperitenone	96
21	25.392	90157630	11,81	16456104	Piperitenone oxide	95
22	25.713	760063	0,18	202118	Methylacetophenone	92
23	27.233	1180658	0,15	360152	Caryophyllene	93
24	31.647	6639527	0,87	1254093	Spathulenol	92
25	31.897	31981055	4,19	7055512	Caryophyllene oxide	96
26	38.311	1013537	0,13	268245	2-Pentadecanone	91

## 2.2. Antimicrobial effect

MLEO demonstrated varying levels of antimicrobial activity against standard pathogenic bacterial strains (Table 2). It exhibited strong inhibitory activity against gram-negative bacterial strains. While a larger zone of inhibition was determined for gram-positive bacteria, *Enterococcus faecium* ATCC 700211 and *Bacillus cereus* ATCC 14579, a smaller zone was observed for other strains.

Essential oils obtained from *Mentha* species have been reported to exhibit strong antimicrobial activity against antibiotic-sensitive pathogens, such as *Escherichia coli*, *Salmonella typhimurium*, *Micrococcus luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* [9,15-17]. In this context, Mahmoudi et al. (2020) reported that *Mentha longifolia* L. essential oil combined with ciprofloxacin and imipenem reduced the expression of genes encoding the adeABC efflux pump in *Acinetobacter baumannii* clinical strains isolated from intensive care units, while enhancing its antimicrobial effect [18]. Shahverdi et al. (2004); demonstrated that a combination of different doses of piperitone isolated from *Mentha longifolia* L. against nitrofurantoin-resistant *Enterobacteriaceae* strains reduced nitrofurantoin resistance in bacteria [19]. Iraj et al. (2020) reported that piperitone exhibited moderate antifungal activity in their study investigating the antifungal activities of monoterpenes against *Candida* species [20]. In agreement

with the literature, MLEO exhibited antimicrobial activity against gram-negative and gram-positive pathogenic bacteria in the present study. While an inhibition zone greater than 10 mm formed against gram-negative bacteria, the zone of inhibition against gram-positive bacteria was variable in size. Research has shown that gram-negative bacteria are more resistant to essential oils than gram-positive bacteria. This has been attributed to the difference in the cell wall structures of gram-positive and gram-negative bacteria. The lipoprotein outer membrane of gram-negative bacteria and increased efflux pump expression play an important role in the resistance of gram-negative bacteria to essential oils [21]. The gram-negative bacterium *Acinetobacter baumannii*, responsible for skin and soft tissue infections, has developed resistance to a significant proportion of commercial antimicrobials, including penicillins, cephalosporins, aminoglycosides, fluoroquinolones, and sulbactam [22]. In the present study, MLEO created a strong zone of inhibition against *Acinetobacter baumannii*, which is capable of developing resistance to antimicrobials using both enzymatic and non-enzymatic pathways. Notably, MLEO has the potential for use in combination with other antimicrobials in skin infections that are difficult to treat due to antimicrobial resistance.

**Table 2.** Inhibition zones of MLEO against selected pathogens

Bacteria Strains	MLEO 10 µg/mL	P10	CN10	E15	AMP10	VA30	DA2	KF30
<i>Acinetobacter baumannii</i> BAA-1605	15 mm	-	-	-	-	-	-	-
<i>Escherichia coli</i> BAA-2523	24 mm	-	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i> ATCC 9027	11 mm	7 mm	-	9 mm	-	-	-	-
<i>Methicillin-resistant Staphylococcus aureus</i> ATCC 67106	8 mm	9 mm	12 mm	-	-	9 mm	-	15 mm
<i>Enterococcus faecium</i> ATCC 700211	17 mm	-	6 mm	-	9 mm	-	-	-
<i>Streptococcus salivarius</i> ATCC 13419	8 mm	15 mm	6 mm	-	12 mm	8 mm	7 mm	8 mm
<i>Streptococcus mutans</i> ATCC 35668	9 mm	21 mm	13 mm	10 mm	16 mm	14 mm	11 mm	24 mm
<i>Enterococcus faecalis</i> ATCC 49452	8 mm	8 mm	6 mm	8 mm	11 mm	8 mm	7 mm	8 mm
<i>Bacillus cereus</i> ATCC 14579	14 mm	22 mm	14 mm	10 mm	16 mm	11 mm	9 mm	26 mm

*Mentha longifolia* L. essential oil (MLEO), Penicillin G (P), Gentamicin (CN), Erythromycin (E), Ampicilin (AMP), Vancomycin (VA), Clindamycin (DA), Cephalothin (KF)

### 2.3. Cell culture dose studies

The cytotoxic potential of MLEO was evaluated using the MTT test. Doses of 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2.5 µg/mL, 5 µg/mL, and 10 µg/mL increased the viability of PCS-201-01 cells compared with the DMSO group. On the other hand, the viability level of the cells at a dose of 20 µg/mL was lower than that of the control and DMSO groups and exhibited a cytotoxic effect. A statistically significant difference was reported for all doses compared with the DMSO group (Figure 2, p<0.05 and p<0.001).

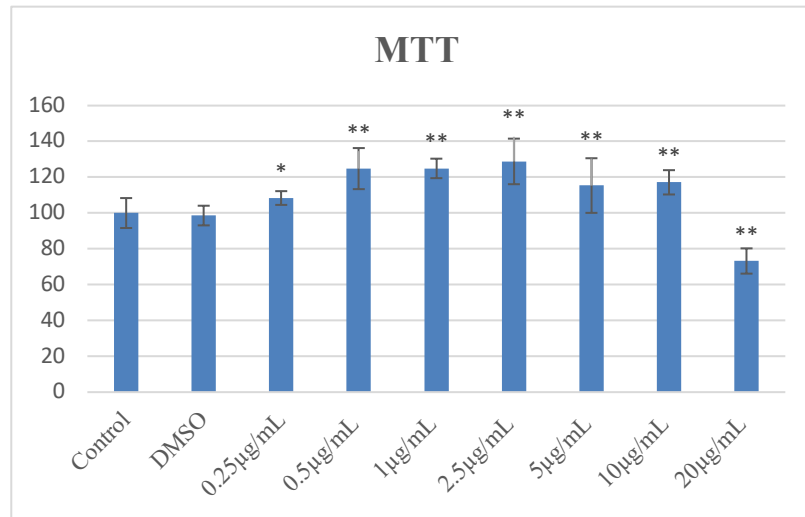


Figure 2. The effect of MLEO doses on the viability of PCS-201-01 cells  
Statistical significance compared to the DMSO group. \*p<0.05 \*\*p<0.001

#### 2.4. Scratch wound healing

A scratch wound healing test was performed to define the proliferation and migration potential of effective doses determined using the MTT test. All treatment doses significantly increased mechanically induced wound line proliferation and fibroblast migration compared to the control group (Figure 3 and Figure 4).

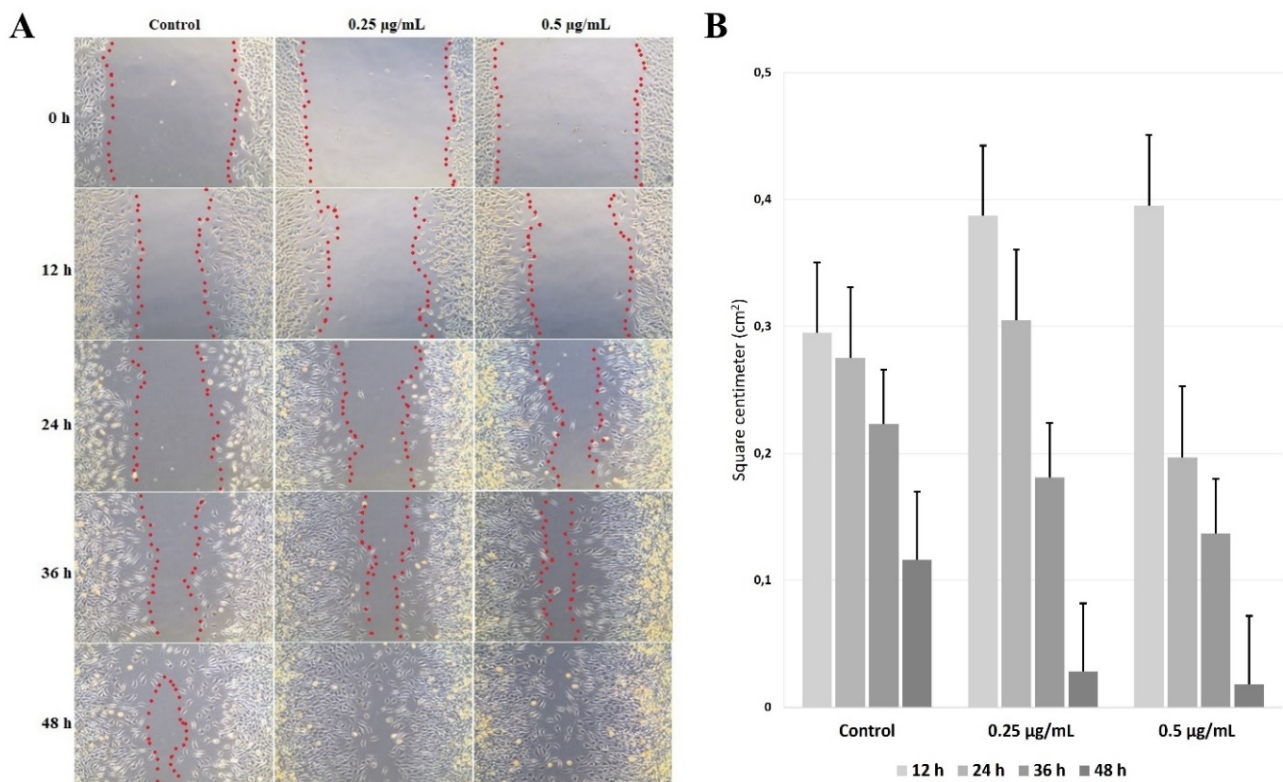
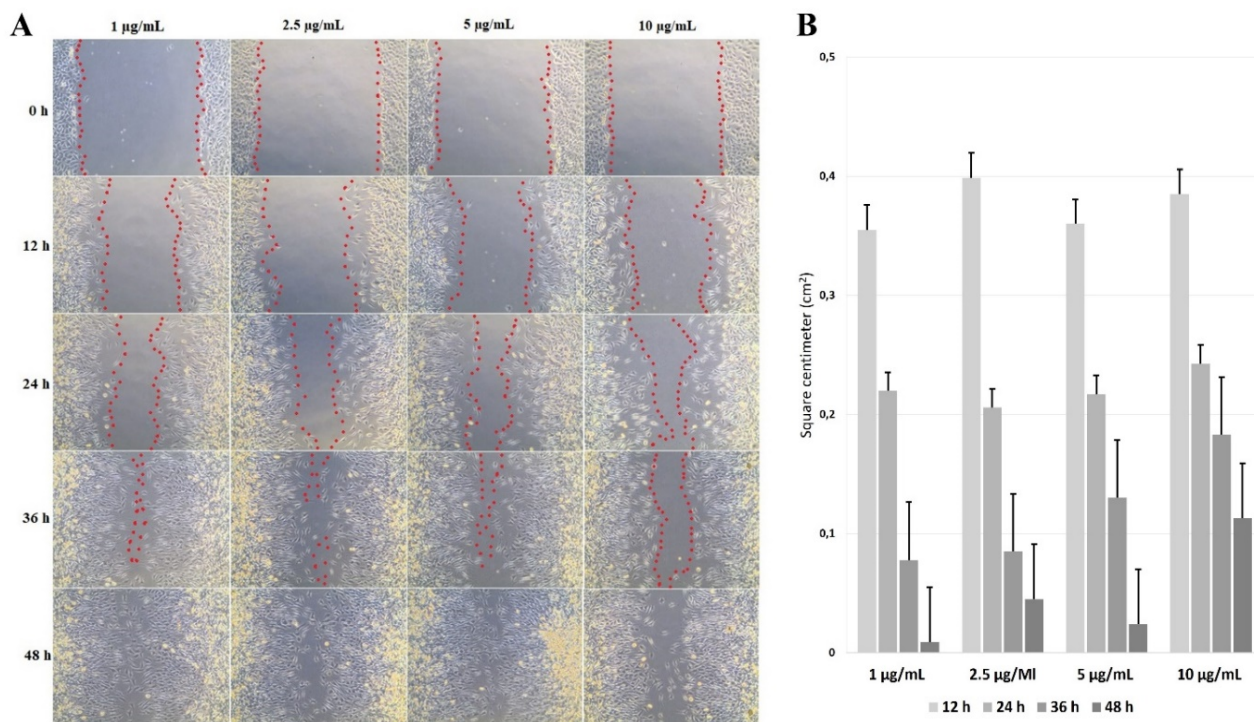


Figure 3. Scratch wound healing assay control, 0.25µg/mL and 0.5µg/mL





**Figure 4.** Scratch wound healing Assay 1 µg/mL, 2.5 µg/mL, 5 µg/mL and 10 µg/mL

Studies investigating the effect of MLEO on wound healing are limited in number. One study, the effect of essential oils obtained from *Mentha longifolia* L. subspecies on wound healing in mice with second-degree burn wounds was investigated. It has been reported that it provides more effective wound healing than the control drug silver sulfadiazine after 21 days of wound follow-up. However, data on wound healing in this study have been limited to histopathological analyzes [23].

The bioactive properties of essential oils are due to their phytochemicals. MLEO contains a large number of active ingredients, and some of which have been proven to be effective in wound healing. Mohammed et al. (2022) reported that serum IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels decreased and accelerated wound healing in rats with first-degree burn wounds; treated with an ointment containing Eucalyptol (1,8-Cineole) [24]. Jiji et al. (2019) on the other hand, reported higher epithelialization and angiogenesis formation in the histopathological examination of mice with third-degree burn wounds that they treated using bacterial cellulose hydrogel containing thymol [25]. Gushiken et al. (2022) treated rats with excision wounds with a gel containing 1%  $\beta$ -caryophyllene have been informed that healing increased in the wound area through antioxidant, anti-inflammatory, wound contraction, re-epithelialization, and remodeling mechanisms [26].

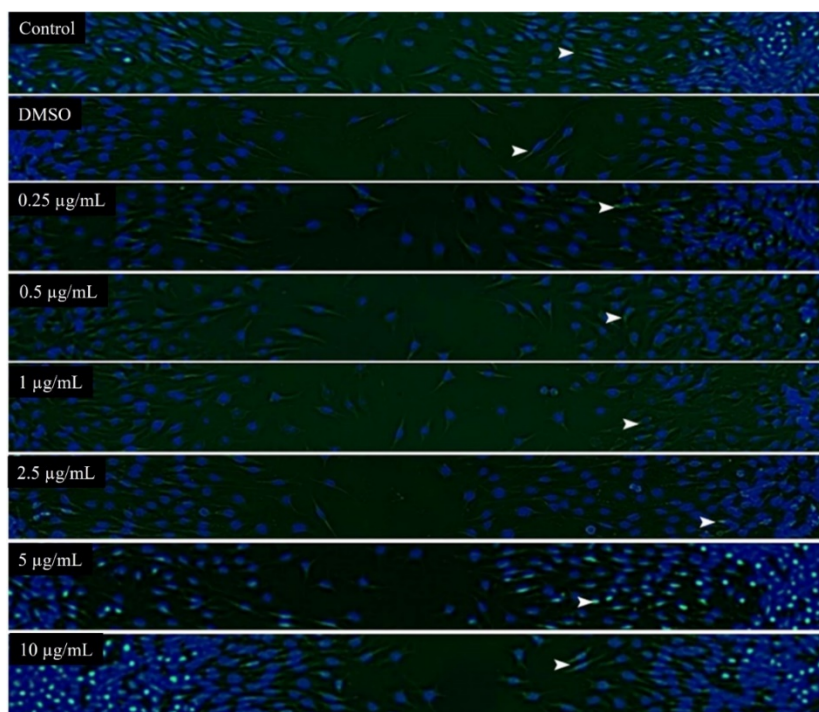
Studies on experimental wound models in the literature, essential oils have been used rather than their components. A 99.73% healing level has been reported in rats with a full-thickness wound model treated with topical application of *Mentha piperita* essential oil, with TGF- $\beta$  expression also increasing in the treatment group [27]. TGF- $\beta$  plays an active role in the inflammation, angiogenesis, re-epithelialization, and connective tissue regeneration stages of wound healing. However, recent studies suggest that while TGF- $\beta$  levels increase in the early phases of wound healing, they should then decrease in the remodeling phase. Studies have reported that itching, tenderness, and scar formation in the wound area may occur due to an increase in TGF- $\beta$  levels during wound healing [28,29]. In the present study, and consistent with the current literature, moderate TGF- $\beta$  expression was observed in groups with faster wound closure. A statistically significant difference was detected between the groups in terms of immunofluorescent staining with Bek (FGF 2), IGF and TGF- $\beta$  ( $p < 0.001$ ) (Table 3).

**Table 3.** Statistical differences in Bek, IGF and TGFβ immunofluorescence staining

Groups	BEK (FGF 2)	IGF	TGFβ
Control	1,16±0,40 <sup>a</sup>	0,33±0,40 <sup>a</sup>	0,16±0,40 <sup>a</sup>
DMSO	1,00±0,00 <sup>a</sup>	0,33±0,40 <sup>a</sup>	0,33±0,40 <sup>a</sup>
0.25 µg/mL	0,83±0,40 <sup>a</sup>	0,16±0,40 <sup>a</sup>	0,16±0,40 <sup>a</sup>
0.5 µg/mL	1,16±0,40 <sup>a</sup>	0,33±0,40 <sup>a</sup>	0,16±0,40 <sup>a</sup>
1 µg/mL	0,83±0,40 <sup>a</sup>	0,16±0,40 <sup>a</sup>	1,16±0,40 <sup>b</sup>
2.5 µg/mL	1,16±0,40 <sup>a</sup>	0,16±0,40 <sup>a</sup>	1,00±0,00 <sup>b</sup>
5 µg/mL	1,66±0,51 <sup>b</sup>	1,83±0,40 <sup>b</sup>	1,00±0,00 <sup>b</sup>
10 µg/mL	1,83±0,40 <sup>b</sup>	2,66±0,51 <sup>c</sup>	0,83±0,40 <sup>b</sup>

a; mild b; moderate, c; severe, p<0.001

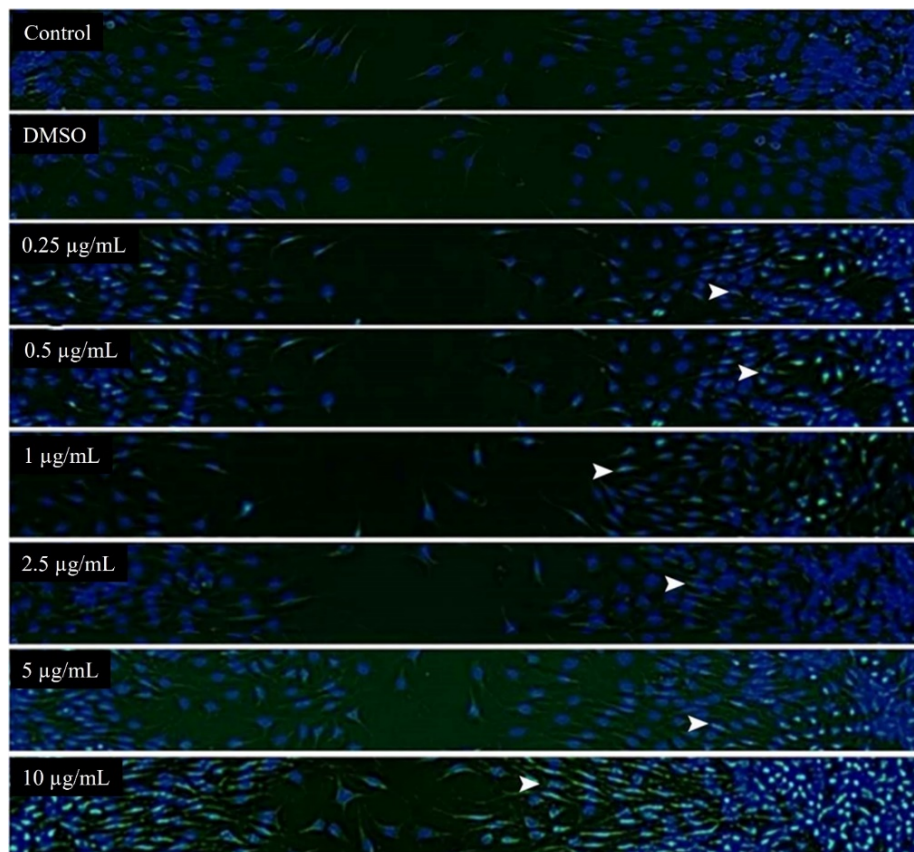
Growth factors interact with proinflammatory cytokines during wound healing. FGF2, TGF-β and IGF, which are produced by human dermal fibroblast cells, play important roles in different stages of wound healing [28]. Studies have shown that FGF2 promotes epithelialization and increases keratinocyte motility and the migration of fibroblasts during re-epithelialization [30]. Researchers have reported that *Mentha piperita* essential oil promoted wound healing and reduced FGF2 expression after healing in an infected wound model in mice [12]. Consistent with the wound closure and inflammation data in this study, FGF2 expression was higher at 5 µg/mL and 10 µg/mL doses than in the other groups. While mild positivity was detected in the first four dose groups in which control, DMSO, and drug applications were employed in fluorescence staining performed with Bek, moderate positivity was detected in the last two groups, those receiving 5 µg/mL and 10 µg/mL doses. In terms of staining with IGF, no significant positivity was detected in the control and DMSO groups. However, while mild positivity was detected in the first four dose groups in the treatment group, moderate fluorescence positivity was observed in the 5 µg/mL group, and severe fluorescence positivity in the 10 µg/mL dose group. The positivity observed in staining with TGF-β was lower than that seen in Bek and IGF. While no significant fluorescence positivity was detected in the control and DMSO 0.25 µg/mL and 0.5 µg/mL groups, mild positivity was observed in the 1 µg/mL and higher dose groups (Figure 5, Figure 6, and Figure 7).



Mild expression in the control, DMSO, 0.25 µg/mL, 0.5 µg/mL, 1 µg, 2.5 µg/mL groups, and moderate expression in the 5 µg/mL and 10 µg/mL groups (arrowhead).

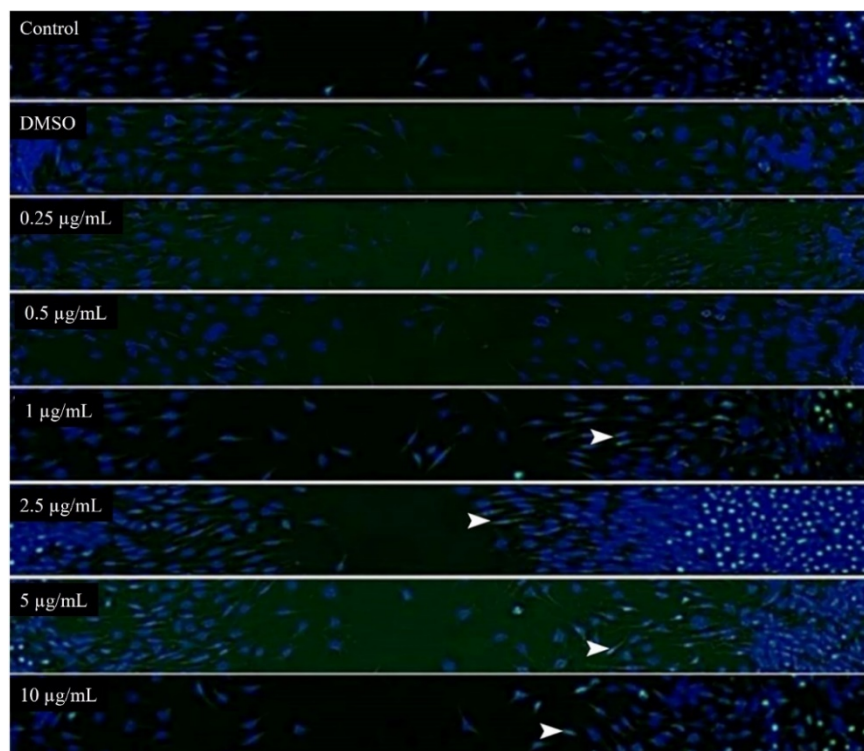
**Figure 5.** Bek (FGF2) immunofluorescence positivity





Negative expression was detected in the control and DMSO groups, mild expression in the 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2.5 µg/mL groups, moderate expression in the 5 µg/mL group, and severe expression in the 10 µg/mL group (arrowhead).

**Figure 6.** IGF immunofluorescence positivity



Negative expression was detected in the control, DMSO, 0.25 µg/mL and 0.5 µg/mL groups, and mild expression in the 1 µg/mL, 2.5 µg/mL, 5 µg/mL and 10 µg/mL groups (arrowhead).

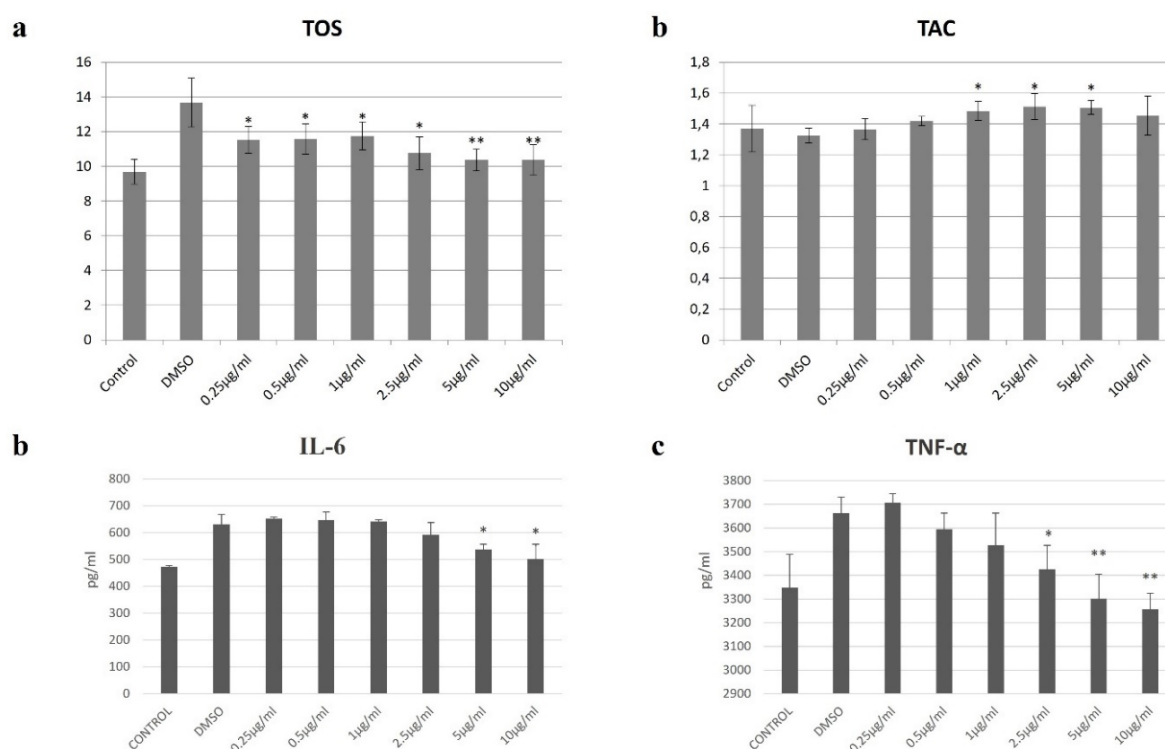
**Figure 7.** TGFβ immunofluorescence positivity



IGF plays an active role in wound healing. Studies show that it regulates the inflammatory response, especially while increasing proliferation and cell migration [31]. Another study using *Mentha piperita* essential oil reported that it increased IGF expression in healthy skin tissue and accelerated hair growth after four weeks of topical application [32]. In the present study, IGF expression was high at doses of 5 µg/mL and 10 µg/mL. The lower levels of IL-6 and TNF-α at these doses than in the DMSO and other treatment groups was associated with regulation of the inflammatory response.

## 2.5. Biochemical data

Compared with the DMSO group, TOS decreased in a dose-dependent manner in the treatment groups, and a statistically significant difference was observed between the treatment groups. Antioxidant capacity levels, on the other hand, yielded results consistent with the oxidant status. However, a statistically significant difference was determined only in the groups receiving 1 µg/mL, 2.5 µg/mL, and 5 µg/mL MLEO doses. Consistent with antioxidant capacity, the proinflammatory cytokines IL-6 and TNF-α decreased as the doses increased (Figure 8). A statistically significant difference was observed only at higher doses ( $p < 0.05$  and  $p < 0.001$ , respectively).



Statistical significance compared to DMSO group, \* $p < 0.05$ , \*\* $p < 0.001$

**Figure 8.** Biochemical analysis results

The antioxidant activity of essential oils has been associated with the dose applied and the oxygenated sesquiterpene content [5]. Studies using *in vitro* tests have confirmed that MLEO exhibits an antioxidant effect [32-34]. A few *in vivo* studies have reported that MLEO exerts especially antioxidant effect in sepsis models [35,36]. MLEO has also been shown to exhibit powerful radical scavenging activity using DPPH and β-carotene bleaching tests. Researchers have reported decreased prostaglandin E2 (PGE2) and cyclooxygenase 2 (COX-2) expression in the liver tissues of rats treated with MLEO following induction of a sepsis model. In that model of sepsis induced by cecal ligation and puncture, the authors attributed the low level of liver injury observed in the treatment group to the antioxidant and antibacterial effects of MLEO [35]. In the present study, total oxidant levels decreased in a dose-dependent manner in the medium obtained from the cell culture wound model. However, antioxidant capacity increased at higher doses. Studies involving essential oils of different plant species have reported that essential components of MLEO, such as piperitone, piperitenone, piperitenone oxide, caryophyllene oxide, and 1,8-cineole, also exhibit antioxidant effects [37-39].

Although the anti-inflammatory effect of essential oils has been described as frequently due to their antioxidant properties, detailed studies have revealed that the situation is actually rather more complex. Essential oils generally exert their anti-inflammatory effects by inhibiting arachidonic metabolism, cytokine

production, and the expression of proinflammatory genes [40]. Arachidonic acid is released by the cell membrane in line with the inflammatory response and is metabolized by cyclooxygenase (COX) or lipoxygenase (LOX). Studies have confirmed that MLEO exhibits an anti-inflammatory effect by suppressing COX-2 expression following sepsis induction in experimental animals [35,40]. Proinflammatory cytokines are produced immediately after skin injuries. These, including TNF- $\alpha$ , IL-6, and IL-1, participate in both the inflammatory phase (by stimulating progenitor cells and immune cells) and the epithelialization phase (promoting cell proliferation and differentiation). The level of proinflammatory cytokines then decreases following wound healing. However, the overproduction of proinflammatory cytokines results in the formation of a systemic response and delayed wound healing [41]. In the present study, IL-6 and TNF- $\alpha$  levels decreased as MLEO dosages increased compared to the DMSO group 48 h after the establishment of the in vitro wound model. The wound line closed faster, and the cell density was higher in these groups. In addition to its antioxidant effect, MLEO is also thought to contribute to wound healing by regulating the production of proinflammatory cytokines.

### 3. CONCLUSION

MLEO exhibited an antimicrobial effect against nine antibiotic-susceptible pathogen strains in this study. Non-cytotoxic doses of MLEO in human fibroblast cells increased cell proliferation and migration in the *in vitro* scratch wound healing assay. Biochemical and immunohistochemical methods confirmed that it possesses antioxidant and anti-inflammatory properties and is supportive of wound healing. Its antimicrobial effect and promotion of wound healing suggests that the essential oil of *Mentha longifolia* L. has substantial potential in terms of the development medical products. However, further *in vivo* studies are needed for it to take place in medical use.

### 4. MATERIALS AND METHODS

#### 4.1. Plant collection

*Mentha longifolia* L. collected from Alanya/Antalya in Turkey in August 2020 (the flowering period) was dried in a cool, shady environment. The plants were kept in closed containers to avoid losing their aromatic properties. The taxonomic identification of plant material was confirmed by Prof. Dr. Meryem Sengul Koseoglu, a senior plant taxonomist in the Biology Department of Atatürk University, Erzurum, Turkey. A voucher specimen has been deposited at the Atatürk University Department of Biology herbarium (ATA Herbarium 10110).

#### 4.2. Essential oil extraction

Once the aerial parts (leaves and flowers) of *Mentha longifolia* L. had been dried and powdered, they were subjected to water distillation for 3-4 hours at 97°C in a Clevenger-type apparatus (Thermal Laboratory Equipment, Turkey). The resulting MLEO was then stored at +4°C for further study [42].

#### 4.3. GC/MS analysis

Gas chromatography/mass spectrometry GC/MS analysis was carried out at the Agri Ibrahim Cecen University Central Research and Application Laboratory. Analysis was performed using a combined system of gas chromatography and mass spectrometry (Shimadzu QP2010). Chromatographic separation was carried out with a capillary column (Teknokroma TRB-5MS Capillary 30 m  $\times$  0.25 mm, 0.25  $\mu$ m). The inert gas (He, purity 99.999%) flow was 0.73 mL min<sup>-1</sup>, the injection volume was 1.0  $\mu$ L, and the injection block temperature was set at 250°C. The split mode was used for injection, and the split ratio was set to 60:1. Electron impact was used as an ionizing technique, and the scanning range of the mass analyzer was adjusted to 50-600 *m/z*.

#### 4.4. Antimicrobial activity

Nine pathogenic bacterial strains, methicillin-resistant *Staphylococcus aureus* ATCC 67106, *Acinetobacter baumannii* ATCC BA1609, *Enterococcus faecium* ATCC 700211, *Escherichia coli* ATCC BAA-2523, *Streptococcus salivarius* ATCC 13419, *Pseudomonas aeruginosa* ATCC 9027, *Streptococcus mutans* ATCC 35668, *Enterococcus faecalis* ATCC 49452, and *Bacillus cereus* ATCC 14579, were used to evaluate the antimicrobial effect of MLEO. In order to determine this activity, 12-h bacterial suspensions inoculated into Mueller-Hinton broth medium were adjusted to 0.5 McFarland standard turbidity. MLEO was applied at doses of 5  $\mu$ g/mL and 10  $\mu$ g/mL

using the agar well diffusion and disk diffusion methods. The antibacterial effect was determined by measuring the inhibition zone around the wells after 12 h of incubation at 37°C [43].

#### 4.5. Cell culture dose studies

Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test on normal human fibroblast cells (PCS-201-01, ATCC, USA) maintained in Dulbecco's modified Eagle's medium high glucose (DMEM-HG) supplemented with 10 mL/L penicillin-streptomycin and 10% fetal bovine serum. PCS-201-01 cells were cultured and kept under an atmosphere of 5% CO<sub>2</sub> and 95% air in an incubator at 37°C. Cells were grown in 96-well flat-bottom plates at a density of approximately  $2 \times 10^5$  cells/well and incubated until 80% confluence was reached. After the medium had been removed from the wells, it was washed with phosphate-buffered saline (PBS). MLEO prepared separately in DMEM-HG at doses of 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, and 20 µg/mL was added to each well at 100 µL. Once the cells had been incubated for an additional 24 h, the treated mixtures were removed and washed with PBS to remove any chemical residues. A 10 µL aliquot of MTT (5 mg/mL in PBS) reagent was added to each well and incubated for a further 4 h at 37°C. Finally, formazan crystals were dissolved in 90 µL of dimethyl sulfoxide (DMSO), and absorbance was measured on a ELISA plate reader (Multiskan™ GO Microplate spectrophotometer reader) at 450 nm. All experiments were performed in triplicate, the mean values for all experiments being subjected to statistical analysis [44].

#### 4.6. Scratch wound healing assay

The cell culture scratch wound healing assay was performed to examine cell migration and proliferation ability relative to treatments. PCS-201-01 cells were grown in DMEM-HG supplemented with 10% FBS and 10 ml/L penicillin-streptomycin. A total of  $2 \times 10^5$  cells/well were cultivated in 24-well cell culture plates and incubated for 24 h until the wells reached 70-80% density in a monolayer. The cellular monolayer was drawn off with a 200 µL pipette tip, without changing the medium, and an artificial linear wound was created. The medium was removed from the wells and gently washed with PBS to remove any dead/unbound cells. Fresh medium prepared with MLEO at doses of 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2.5 µg/mL, and 5 µg/mL was added to the wells at 400 µL. The best-recorded doses from the cell viability assay were selected for this experiment and evaluated in three replicates. Wound size was observed at specific periods during the 0-48 h incubation. Scratch wound closures were observed using a LEICA DFC295 inverted microscope for photograph capture. In order to measure cell migration ability, the width of the scratch area was photographed at 0 h, 12 h, 24 h, 36 h, and 48 h time intervals. The experiment was terminated at the 48 h, when the treatment groups closed the wound line [45].

#### 4.7. Immunohistochemical staining

Cells were fixed with methanol for 5 min at -20°C and washed with PBS. They were then incubated with PBS containing 0.1% Triton X-100 at room temperature for 15 min. After washing, they were incubated with PBS containing 2% BSA for 60 min at room temperature. After rewashing, monoclonal anti-Bek (Santa Cruz, Catalog no. sc-6930), monoclonal anti-IGF (Santa Cruz, Catalog no. sc-518040), and monoclonal anti-TGF-β (Santa Cruz, Catalog no. sc-130348) were incubated overnight at +4°C at a dilution rate of 1/200 with primary antibodies. Finally, 4',6-diamidino-2-phenylindole (DAPI) was dropped onto the washed cells and examined under a fluorescence microscope. Fluorescence positivity observed near the wound line was evaluated semiquantitatively as absent (-), mild (+), moderate (++), or severe (+++). The data were analyzed on SPSS version 20.00 [46].

#### 4.8. Biochemical analysis

Total antioxidant capacity (TAC) and total oxidant status (TOS) activities were measured from the 48 h medium obtained from the wound model analysis, using TAC and TOS commercial kits produced by Rel Assay Diagnostics® (Gaziantep, Turkey). IL-6 and TNF-α levels were determined using the Elabscience® (United States) off-the-shelf assay procedure. The results were analyzed on SPSS version 20.00 software [47].

#### 4.9. Statistical analysis

Biochemical analyses were performed using one-way analysis of variance (ANOVA) with post hoc Tukey's test (IBM SPSS 22.0) ( $p < 0.05$ ). Data are shown as mean  $\pm$  standard deviation (SD). Differences between the groups in terms of immunohistochemical staining were determined using the Kruskal–Wallis test, one of the nonparametric tests, and the Mann–Whitney U test was applied for the group that created the difference ( $p < 0.05$ ) [46,47].

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