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RESEARCH ARTICLE

Wound Healing Mechanisms of *Medicago sativa* Honey: Antioxidant, Anti-inflammatory, Antibacterial and Phytochemical Characterisation

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Abstract

Background: Honey has long been used in traditional medicine for wound healing. Its therapeutic properties vary depending on botanical and geographical origin. **Aim:** To evaluate the physicochemical characteristics, polyphenolic composition, antioxidant potential, antibacterial activity, and wound healing effects of Palestinian *Medicago sativa* (alfalfa) honey. **Methods:** Polyphenols were profiled by HPLC. Antioxidant capacity was assessed by Total Phenolic Content (TPC) and DPPH radical scavenging assays. Anti-inflammatory activity was determined by Nitric Oxide (NO) production in LPS-activated THP-1 macrophages. Wound healing potential was tested via HaCaT keratinocyte proliferation and scratch migration assays. Antibacterial effects were evaluated against Gram-positive and Gram-negative strains. **Results:** HPLC revealed a diverse polyphenolic profile dominated by ellagic acid (23.51 mg/g), pinocembrin, and myricetin, with a TPC of 47.37 mg/g. Antioxidant assays confirmed high phenolic levels (327.5 ± 6.95 mg GAE/100 g) and strong radical scavenging activity (DPPH IC_{50}: 12.33 ± 0.68 mg/mL). Alfalfa honey significantly reduced NO production in macrophages, enhanced keratinocyte proliferation at low concentrations, and promoted migration at 1-2 mg/mL, while higher doses showed cytostatic effects. Antibacterial assays demonstrated broad-spectrum activity, particularly against *Bacillus subtilis* and *Klebsiella pneumoniae*. **Conclusion:** Palestinian *M. sativa* honey exhibits potent antioxidant, anti-inflammatory, antibacterial, and wound healing properties. Its high ellagic acid content, dose-dependent effects on keratinocyte proliferation, and broad antibacterial activity highlight its potential as a natural therapeutic agent in wound management.

Major Findings: Palestinian alfalfa honey shows exceptional wound-healing potential through strong antioxidant, anti-inflammatory, and antibacterial activities, largely attributed to its high ellagic acid and polyphenolic content.

Keywords: Antioxidant Activity, HaCaT Cells, *Medicago sativa* Honey, Polyphenols, Wound Healing

1. Introduction

Wound healing is a complex, multifaceted process involving tightly regulated phases: hemostasis, inflammation, proliferation, and remodeling. Chronic wounds—such as diabetic foot ulcers, venous leg ulcers, and pressure ulcers—pose a major healthcare burden worldwide, often complicated by infections,

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prolonged inflammation, and impaired tissue regeneration[1,2]. Traditional treatments, including antibiotics and synthetic dressings, face limitations due to increasing antimicrobial resistance, side effects, and insufficient efficacy in stimulating tissue repair[3]. These challenges highlight the need to explore alternative or complementary wound care agents with proven bioactivity.

Honey has been employed for centuries in traditional healing practices due to its diverse therapeutic effects. It exerts a broad spectrum of bioactivities, including immunomodulatory, antimicrobial, antioxidant, anti-inflammatory, and wound-repair functions. The biological efficacy and chemical composition of honey are strongly influenced by the floral source and geographic region from which it is derived. Typically, honey is composed predominantly of sugars (mainly d-glucose and d-fructose), small amounts of water, and a variety of minor constituents such as amino acids, proteins, lipids, vitamins, minerals, and a complex mixture of phytochemicals including polyphenols, flavonoids, terpenoids, and volatile compounds.

Among the wide array of honey types, monofloral honeys—originating primarily from the nectar of a single plant species—have drawn increasing scientific interest due to their distinctive chemical fingerprints and targeted therapeutic properties. A notable example is honey derived from *M. sativa* (commonly known as alfalfa or lucerne), a leguminous plant recognised for its high content of bioactive secondary metabolites. When bees forage on alfalfa blossoms, the resulting honey incorporates specific phytochemicals present in the plant's nectar. Recent investigations have highlighted the richness of alfalfa honey in polyphenolic compounds such as ellagic acid, pinocembrin, and myricetin—molecules widely acknowledged for their roles in oxidative stress reduction, antimicrobial defense, and inflammatory regulation[4,5]. These properties suggest a strong potential for use in natural therapeutic formulations.

Furthermore, the antioxidant profile of alfalfa honey may facilitate tissue regeneration and cellular repair, processes that are essential in the context of wound management. Its ability to modulate inflammation and oxidative damage also lends support to its proposed anticancer potential[4]. However, while honey's general wound-healing benefits are recognised, the precise cellular and molecular mechanisms—particularly those specific to alfalfa honey—remain incompletely characterised. Existing studies provide limited data on how its bioactive components interact with signaling pathways involved in inflammation, oxidative stress, and cell proliferation. This gap motivates the present investigation.

Natural products like honey have gained renewed interest as alternative or complementary therapies for wound care. Honey's unique chemical composition—comprising sugars, organic acids, enzymes, phenolic compounds, and antimicrobial peptides—provides synergistic antibacterial, antioxidant, anti-inflammatory, and tissue-regenerative effects[6,7]. These multifactorial actions help reduce bacterial colonisation, modulate oxidative stress, regulate immune responses, and promote cell proliferation and migration critical for wound closure[8]. Recent clinical and preclinical studies underscore honey's efficacy in accelerating wound healing, reducing inflammation, and improving patient outcomes in both acute and chronic wound models[9,10]. Moreover, honey's biocompatibility, low cost, and ease of application make it especially valuable for use in resource-limited settings and as an adjunct in modern wound care products such as hydrogels and dressings[11].

Honey has reemerged as a valuable therapeutic agent in modern wound care due to its antimicrobial, anti-inflammatory, and regenerative properties. Its efficacy has been increasingly supported by scientific evidence, particularly in the context of managing chronic wounds, including diabetic foot ulcers, venous leg ulcers, and pressure sores. A recent meta-analysis confirmed that honey dressings significantly accelerate healing, reducing wound closure time by an average of 17 days compared to standard care options[12].

The wound healing process involves four overlapping phases: hemostasis, inflammation, proliferation, and remodeling. Honey supports each of these phases through several mechanisms. Its high sugar content and low water activity create a hyperosmolar environment that promotes lymphatic drainage and inhibits microbial growth. Additionally, the natural acidity of honey (pH 3.2-4.5) enhances oxygen release in tissues and promotes fibroblast function[13].

Honey contains a range of bioactive compounds, including hydrogen peroxide (formed enzymatically upon dilution), phenolic acids, flavonoids, and

bee-derived peptides, all of which contribute to its antibacterial, antioxidant, and anti-inflammatory properties. These compounds act in synergy to modulate cellular oxidative stress and inflammatory signaling[14]. For example, honey has been shown to suppress iNOS and IL-1β expression while activating NRF2/HO-1 antioxidant pathways, which facilitate tissue regeneration and remodeling[13]. Recent *in vitro* studies have demonstrated that honey stimulates keratinocyte proliferation and migration, enhances collagen production, and downregulates pro-inflammatory cytokines in human fibroblast and macrophage models. Romanian honey varieties, for instance, showed dose-dependent activation of cell repair processes that strongly correlated with their total phenolic and flavonoid content[15]. However, these mechanistic insights remain limited for many honey types, including Palestinian alfalfa honey, underscoring the need for further targeted studies.

Beyond its use as a raw dressing, honey is now incorporated into advanced wound care systems, including hydrogels, nanofiber scaffolds, and bioactive films. These engineered materials facilitate sustained release of honey's active compounds, maintain a moist healing environment, and reduce bacterial burden—offering improved wound closure outcomes in both preclinical and clinical settings[16,17].

Given its multifaceted bioactivity, honey—especially when sourced from medicinal plants—continues to attract attention as a biocompatible, natural therapeutic with growing relevance in tissue engineering and regenerative medicine. Therefore, the present study aimed to comprehensively evaluate the physicochemical properties, polyphenolic profile, antioxidant capacity, antibacterial effects, and wound healing potential of Palestinian alfalfa honey. Using a combination of chromatographic, biochemical, cellular, and microbiological assays, we sought to elucidate the therapeutic relevance of this honey variety in skin regeneration and infection control. Our results demonstrate that alfalfa honey is rich in phenolic compounds—particularly ellagic acid, pinocembrin, and myricetin—exhibiting potent antioxidant activity and significant antibacterial effects against both Gram-positive and Gram-negative bacteria. Moreover, the honey modulated inflammatory responses in macrophages, enhanced keratinocyte proliferation at low concentrations, and accelerated cell migration in scratch assays. These findings underscore the multifaceted therapeutic potential of Palestinian alfalfa honey and support its integration into natural wound care strategies and bioactive formulations.

2. Materials and Methods

2.1 Honey Sample Source and Storage

The honey sample utilised in this investigation was sourced in 2021 from alfalfa orchards in the Jordan Valley region. Procurement was done through "Honey Spring," a certified vendor based in Tulkarem, Northern West Bank. To ensure preservation, the sample was sealed in metal containers and stored under dry, ambient conditions until further analysis.

2.2 HPLC Profiling of Phenolic Constituents

Phenolic compounds in the honey were identified and quantified using High-Performance Liquid Chromatography (HPLC) based on established methods[18].

2.3 Antioxidant Activity via DPPH

The capacity of the honey to scavenge free radicals was measured using a modified version of the DPPH microdilution assay as previously described[19,20].

2.4 Total Antioxidant Capacity (TAC)

TAC was assessed using the phosphomolybdenum method following the procedure outlined by Prieto *et al.*[21]. Results were expressed as mg ascorbic acid equivalents per gram of honey, and all assays were conducted in triplicate.

2.5 Quantification of Total Phenolics and Flavonoids

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) were determined through colorimetric assays as per established protocols[19,22].

2.6 Evaluation of Antibacterial Properties

The antimicrobial potential of the honey was tested against a panel of bacterial strains: *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC BAA-1026), *Escherichia coli* (ATCC 25922),

Streptococcus pneumoniae (ATCC 49619), *Klebsiella quasipneumoniae* (ATCC 700603), *Haemophilus influenzae* (ATCC 49247), and *B. subtilis* (ATCC 6633), all obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC) were determined via standard broth microdilution assays in 96-well plates[19].

2.7 Culturing of Human Cell Lines

This study employed the HaCaT human keratinocyte cell line (CLS Cat# 300493) and the THP-1 monocytic cell line (ATCC TIB-202). Cells were cultured in DMEM (Biological Industries, Israel) supplemented with 10% fetal calf serum, 1% non-essential amino acids, 1% glutamine, 100 U/mL penicillin, and 10 µg/mL streptomycin. Incubation was carried out at 37°C with 5% CO_2.

2.8 Assessment of Cytotoxic and Cytostatic Effects

To evaluate cytotoxicity, 20,000 HaCaT cells per well were plated in 96-well plates and allowed to adhere for 24 h. Cells were then exposed to honey at concentrations from 0 to 4000 µg/mL for another 24 h, after which cell viability was determined using the MTT assay. For proliferation analysis, 5,000 cells/well were seeded and treated similarly, but incubated for 72 h before conducting the MTT assay as per the protocol by Abu-Farich *et al.*[22].

2.9 *In Vitro* Wound Healing Assay

A scratch (gap closure) assay was conducted using HaCaT cells seeded in 12-well plates at 400,000 cells/well. After 24 h of incubation and reaching confluency, a linear scratch was made using a sterile 200 µL pipette tip. The wells were gently rinsed with serum-free DMEM to remove detached cells. Subsequently, triplicate wells were treated with either a 4 mg/mL honey solution or control medium. Wound closure was monitored and documented at 0, 24, and 48 h.

2.10 Nitric Oxide Inhibition

To assess anti-inflammatory potential, THP-1-derived macrophages were activated with LPS and treated with honey samples. Nitrite accumulation in the medium was measured using the Griess assay, following the method described by[18].

2.11 Statistical Analysis

All experimental data are presented as means ± Standard Deviation (SD). Group comparisons were made using unpaired Student's *t*-tests, with statistical significance set at $p<0.05$.

3. Results and Discussion

Honey has been used for millennia in the main traditional medical systems such as, *Ayurveda*, Chinese, and GrecoArab and Islamic traditional medicine as a trusted remedy for wound healing—valued for its capacity to cleanse, reduce inflammation, and promote tissue regeneration[23]. Alfalfa honey, in particular, has been employed in traditional skin and wound-related therapies. Our experimental findings affirm these traditional uses by demonstrating that Palestinian alfalfa honey exerts multiple woundhealing activities including antioxidant, antiinflammatory, proliferative, and antibacterial effects.

3.1 Physicochemical Properties of Alfalfa Honey

The physicochemical characteristics of the alfalfa honey sample are summarised in Table 1. The water-soluble protein content was found to be 497.5 ± 54.3 mg Eq BSA/100 g, indicating a high protein fraction, which may contribute to its biological activity. Total sugar content was measured at 24.97 ± 4.14 g/100 g, reflecting the natural carbohydrate composition of the honey. The pH value of the sample was 3.28 ± 0.02, consistent with the acidic nature of honey, which contributes to its antimicrobial properties. The Brix index was recorded at 72.6 ± 3.2, corresponding to the total soluble solids and suggesting a high sugar concentration. Moisture content was relatively low, at 18%, which is within the acceptable range for unfermented honey and supports its long-term stability. The electrical conductivity of the sample was 55.6 ± 2.05 µS/cm, classifying it within the range typical for nectar-derived honeys. Finally, the honey was visually characterised as extra light amber, indicating a relatively light-coloured honey type that may be associated with its botanical origin and lower mineral content.

The physicochemical properties of the alfalfa honey sample fall within the ranges established by the Codex Alimentarius and other international standards[23,24]. Its low moisture, acidic pH, and moderate conductivity are characteristic of high-quality nectar honeys. The elevated protein content may indicate enzymatic richness, which can contribute to antioxidant and antimicrobial activity, supporting its therapeutic value[25].

3.2 Antioxidant Properties of Alfalfa Honey

The antioxidant properties of Palestinian alfalfa honey were evaluated through quantification of its Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Total Antioxidant Capacity (TAC), and its DPPH radical scavenging activity (Table 2).

The TPC was found to be 327.5 ± 6.95 mg Gallic Acid Equivalents (GAE) per 100 g of honey, indicating a high concentration of polyphenolic compounds. In parallel, the TFC was measured at 29.37 ± 0.24 mg Quercetin Equivalents (QE) per 100 g, reflecting a moderate level of flavonoid constituents.

The TAC, as assessed by the phosphomolybdenum assay, reached 2.25 ± 0.04 g Ascorbic Acid Equivalents (AAE) per 100 g, further supporting the potent antioxidant potential of the honey. Consistent with these findings, the DPPH assay revealed an IC_{50} value of 12.33 ± 0.68 mg/mL, indicating effective free radical scavenging activity. Lower IC_{50} values denote higher antioxidant efficiency, and this result suggests that alfalfa honey possesses moderate-to-strong antioxidant potential, likely attributable to its rich phenolic and flavonoid composition.

3.3 HPLC Analysis of Phenolic Compounds in Alfalfa Honey

HPLC analysis of alfalfa honey revealed a rich and diverse phenolic profile, with the concentrations of individual compounds summarised in Figure 1 and Table 3. The total quantified polyphenol content reached 47.373 mg/g, which is considerably higher than what has been reported for many other unifloral honeys, where typical phenolic levels range from 2 to 20 mg/g depending on botanical and geographical origins[26,27]. This elevated phenolic concentration reflects the unique phytochemical richness of alfalfa honey and may contribute significantly to its biological activities.

Among the identified phenolics, ellagic acid was the most abundant, with a concentration of 23.51 mg/g, accounting for nearly half of the total phenolic content. Ellagic acid is recognised for its strong antioxidant and anti-inflammatory activities and its ability to modulate oxidative stress and support tissue repair, which are crucial in wound healing and cancer prevention[28]. Other major constituents included myricetin (7.99 mg/g), pinocembrin (6.3 mg/g), and p-coumaric acid (6.021 mg/g), all of which are known for their antimicrobial, anticancer, and anti-inflammatory effects[29,30].

Additionally, kaempferol (4.002 mg/g) and pinobanksin (2.00 mg/g) were present in appreciable amounts, while caffeic acid (1.2 mg/g), naringin (1.03 mg/g), and pyrogallol (0.9 mg/g) were detected at moderate concentrations. Quercetin (0.3 mg/g) and gallic acid (0.42 mg/g) were found in lower quantities. These phenolic acids and flavonoids have been widely studied for their free radical scavenging, antioxidant, and anti-inflammatory activities, often

Table 1. Physicochemical characteristics of alfalfa honey. Hydrosoluble protein levels, total sugar levels, pH, brix index, refractive index, electrical conductivity, and colour of the alfalfa honey

Water-soluble proteins	Total sugar	pH	Brix index	Moisture	Conductivity	Colour
497.5±54.3	24.97±4.14	3.28±0.02	72.6±3.2	18	55.6±2.05	Extra light amber

Hydrosoluble Protein (mg Eq BSA/100 g of honey), Total sugar (mg Eq Glu/100 g of honey), Brix scale (%), and Conductivity (µs)

Table 2. Total Phenolic Contents (TPC), Total Flavonoid Contents (TFC), Total Antioxidant Capacity (TAC) and DPPH IC_{50} in Palestinian alfalfa honey

TPC mg Eq GA/100 g	TFC mg Eq Q/100 g	TAC g Eq AA/100 g	DPPH IC_{50} mg/mL
327.5 ± 6.95	29.37 ± 0.24	2.25 ± 0.04	12.33 ±0.68

acting synergistically to enhance honey's therapeutic efficacy[31].

The overall biochemical complexity of alfalfa honey, particularly its high concentration of polyphenols and broad range of bioactive compounds, underlines its potential as a natural therapeutic agent. The specific phenolic composition observed may explain its strong pharmacological activities, including antioxidant, antimicrobial, and wound-healing properties, and supports its candidacy for further development in natural health products.

3.4 Mineral Composition

The elemental profile of alfalfa honey was assessed using inductively coupled plasma optical emission spectrometry (ICP-OES), focusing on both essential and potentially toxic elements (Table 4). The sample exhibited a typical mineral composition for high-quality natural honeys. Potassium was the most abundant macroelement (7.96 mg/L), followed by sodium (7.58 mg/L), calcium (2.93 mg/L), phosphorus (2.38 mg/L), and magnesium (0.93 mg/L). These minerals, naturally derived from the floral and geographical origin, are essential for physiological processes such as enzymatic function and osmoregulation[32,33].

Trace elements including aluminum (0.11 mg/L), silicon (0.19 mg/L), boron (0.034 mg/L), and zinc (0.033 mg/L) were also detected. Although present in low concentrations, these elements may contribute to the nutritional and antioxidant properties of honey, with zinc playing a critical role in immune and repair functions[34]. Notably, heavy metals of toxicological concern—such as lead, cadmium, chromium, cobalt, copper, iron, and nickel—were all below the detection limit (<0.01 mg/L), indicating no contamination from environmental or anthropogenic sources. These values comply with international food safety standards, including those of the Codex Alimentarius and the European Commission, which recommend maximum limits of 0.1 mg/kg for lead in honey[23].

The favorable elemental profile of alfalfa honey confirms its purity, safety, and potential nutritional value, consistent with reports on other unifloral honeys from clean environments[29,35].

3.5 Cytostatic and Cytotoxic Effects of Alfalfa Honey on HaCaT Cells

The effects of alfalfa honey on the proliferation and viability of HaCaT keratinocytes were evaluated across a concentration range of 0 to 16 mg/mL. As shown in

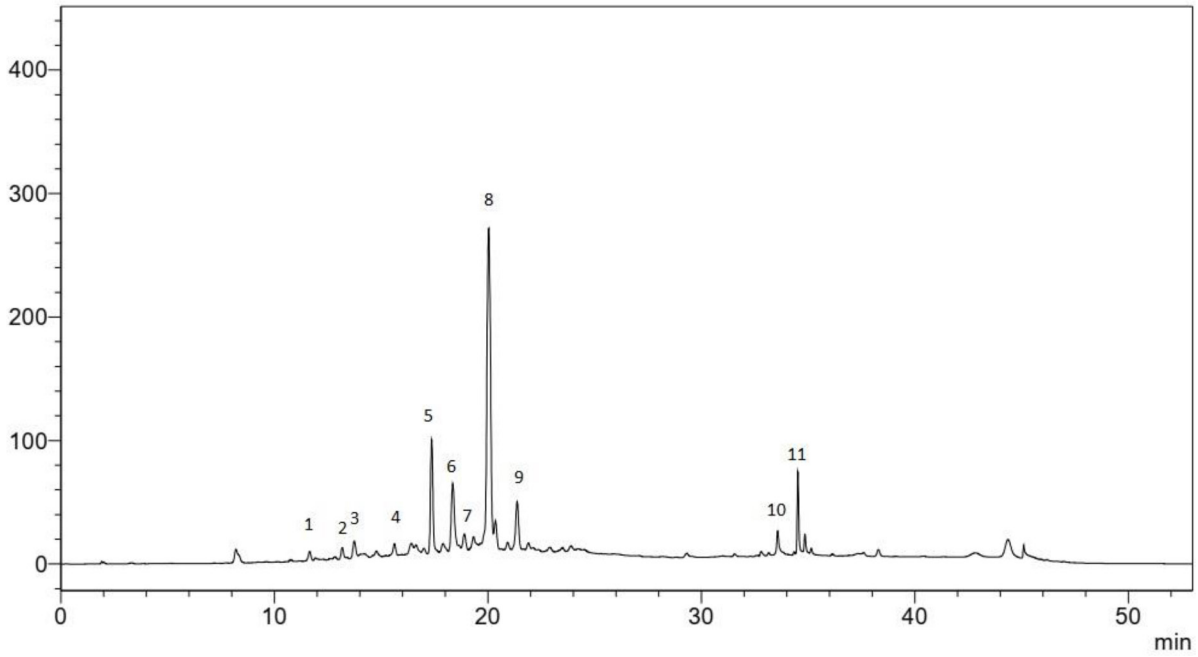

Figure 1. Chromatogram of the quantitative HPLC of alfalfa honey. 1-Gallic acid, 2-Pyrogallol, 3-Naringin, 4-Caffeic acid, 5-Myricetin, 6-P coumaric acid, 7-Quercetin, 8-Ellagic acid, 9-Kaempferol, 10-Pinobanksin, and 11-Pinocembrin.

Table 3. Polyphenolic profile of alfalfa honey. The concentrations (mg/g) of polyphenolic compound determined and Total Polyphenolic Concentration (TPC) in alfalfa honey were determined by quantitative HPLC

Phenolic compounds	Concentration in mg/g
Gallic acid	0.42
Pyrogallol	0.9
Naringin	1.03
Caffeic acid	1.2
Myricetin	7.99
P coumaric acid	6,021
Quercetin	0.3
Ellagic acid	23.51
Kaempferol	4,002
Pinobanksin	2.00
Pinocembrin	6.3
Totals polyphenols	47,373

Figure 2, alfalfa honey exhibited a biphasic cytostatic response, with a notable stimulatory effect on cell proliferation at low concentrations and a gradual reduction in this effect at higher doses.

At 2 mg/mL, a pronounced proliferative response was observed, with cell proliferation reaching approximately 185% of the untreated control, while cell viability remained near 100%, indicating no cytotoxic effects at this concentration. At 4 mg/mL, proliferation remained elevated (~160%), with continued minimal cytotoxicity. As concentrations increased to 8 mg/mL and 16 mg/mL, a decline in both proliferation (~110% and ~80%, respectively) and viability (~95% and ~90%, respectively) was observed, although cytotoxic effects remained modest even at the highest concentration tested.

These findings highlight the concentration-dependent nature of alfalfa honey's biological effects on keratinocytes. The significant enhancement of proliferation at low concentrations suggests a potential regenerative role, particularly relevant in the context of wound healing. Keratinocyte proliferation and migration are vital for re-epithelialisation, the phase of wound healing during which the epidermis is restored[36]. Hence, agents that can stimulate keratinocyte growth without inducing cytotoxicity are of great therapeutic interest.

Table 4. Elemental composition of alfalfa honey

Element	Concentration (mg/L)
Al	0.1095
B	0.0342
Ca	2.9322
K	7.9620
Mg	0.9330
Na	7.5753
P	2.3782
Si	0.1930
Zn	0.0332
Ag, Co, Cr, Cu, Fe, Mo, Ni, Pb, Se, Sn, Sr, V, Mn	< 0.01

This stimulatory effect may be closely linked to the rich polyphenolic composition of alfalfa honey. HPLC analysis revealed a high total polyphenol content of 47.373 mg/g, substantially exceeding the typical range (2-20 mg/g) reported for most unifloral honeys[4,37]. Notably, ellagic acid (23.51 mg/g) was the dominant phenolic compound detected, comprising nearly half of the total polyphenol content. Ellagic acid has well-documented antioxidant and anti-inflammatory properties and plays a crucial role in reducing oxidative stress and supporting tissue regeneration[38]. Such activities may directly or indirectly enhance cellular proliferation by maintaining redox balance and modulating signaling pathways associated with cell cycle progression.

Other abundant polyphenols identified—myricetin, pinocembrin, and p-coumaric acid—are also known for their mitogenic and cytoprotective effects[39,40]. These compounds can activate intracellular pathways (*e.g.*, PI3K/Akt, ERK1/2) involved in cell survival, proliferation, and migration, which may explain the heightened keratinocyte proliferation observed at 2-4 mg/mL honey concentrations. Flavonoids such as kaempferol, naringin, and quercetin, even in moderate amounts, may synergistically enhance this effect through their antioxidant capacity and ability to modulate gene expression linked to wound healing.

Moreover, the absence of significant cytotoxicity, even at higher concentrations, confirms the biocompatibility of alfalfa honey with skin-derived cells, reinforcing its potential for topical applications. The biphasic cytostatic pattern is consistent with hormetic

behaviour, whereby low doses of phytochemicals stimulate beneficial stress responses, while higher concentrations may exert inhibitory effects due to osmotic imbalances or excessive accumulation of reactive compounds[41].

In conclusion, the high polyphenol content of alfalfa honey—especially the presence of ellagic acid and flavonoids—likely contributes to its cytostatic profile, particularly its stimulatory effects on keratinocyte proliferation. This supports its potential as a natural, polyphenol-rich agent for promoting wound healing and tissue regeneration.

3.6 Effect of Alfalfa Honey on HaCaT Cell Migration

The influence of alfalfa honey on HaCaT keratinocyte migration was assessed using a scratch assay at concentrations of 0, 1, 2, and 4 mg/mL over 24- and 48- h intervals. Cell migration was quantified as the percentage of wound closure relative to the untreated control (Figure 3). In control wells, complete wound closure (100%) was observed at both 24 and 48 h, confirming the inherent migratory capacity of HaCaT cells under standard conditions.

Treatment with 1 mg/mL alfalfa honey significantly enhanced cell migration, with wound closure increasing to approximately 115% at 24 h and 145% at 48 h, indicating a strong pro-migratory effect. At 2 mg/mL, migration was comparable to control at 24 h (~100%) and showed a modest increase to ~110% at 48 h, suggesting that this concentration supports or slightly improves cell motility. However, higher concentrations led to a decline in migration: at 4 mg/mL, wound closure dropped to ~70% at 24 h and ~95% at 48 h, suggesting a potential inhibitory effect on cell movement.

These findings demonstrate that alfalfa honey enhances keratinocyte migration in a dose-dependent manner, with optimal activity at low concentrations. Keratinocyte migration is a vital step during re-epithelialisation, a key phase of the wound healing process where cells migrate to cover the wound bed[36]. Enhancing this process is crucial for promoting rapid and efficient wound closure, particularly in chronic or delayed-healing wounds.

The observed migratory enhancement at 1 mg/mL may be attributed to the high content of bioactive polyphenols in alfalfa honey. HPLC analysis revealed a total phenolic content of 47.373 mg/g, with major constituents such as ellagic acid (23.51 mg/g), myricetin, pinocembrin, and p-coumaric acid—all of which are known to modulate key signaling pathways

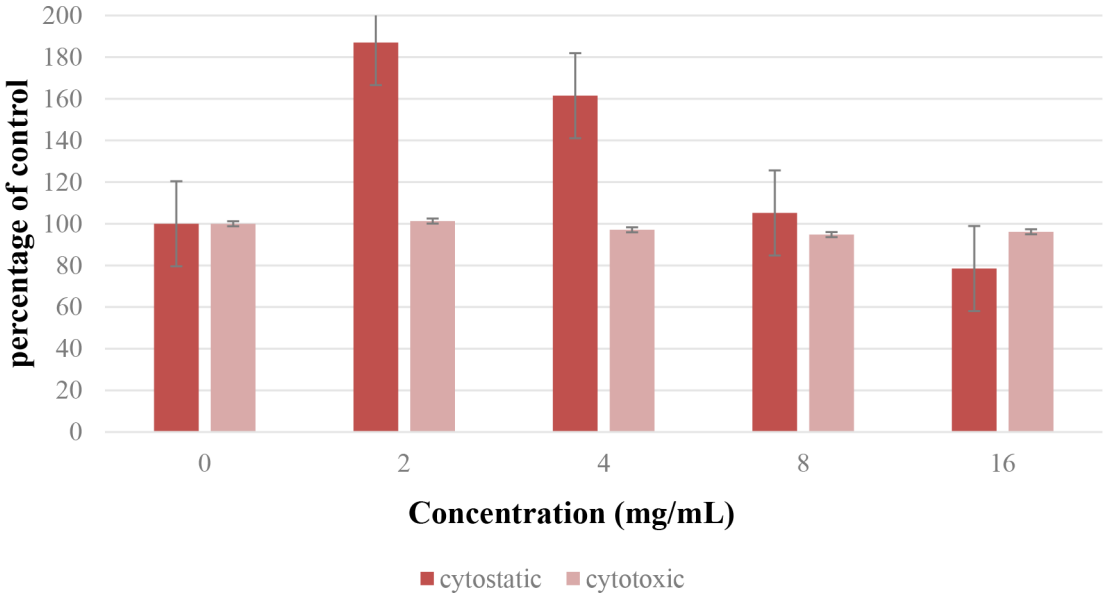

Figure 2. Cytostatic and cytotoxic effects of alfalfa honey on HaCaT cells at various concentrations. HaCaT cells were treated with alfalfa honey at concentrations of 0, 2, 4, 8, and 16 mg/mL. Cytostatic activity indicates the degree of cell proliferation relative to the untreated control, while cytotoxicity reflects the impact on cell viability. Data are presented as a percentage of the control (mean ± SD, n = 3).

involved in cell migration and wound healing[4,42]. Ellagic acid, in particular, has been shown to promote keratinocyte migration through antioxidant and anti-inflammatory mechanisms, including regulation of Matrix Metalloproteinases (MMPs) and modulation of cellular oxidative stress[43].

Furthermore, flavonoids like kaempferol and pinobanksin may influence cytoskeletal reorganisation and cell adhesion dynamics, thereby facilitating directed migration[44,45]. The biphasic pattern observed—with inhibition at higher doses—suggests a hormetic response, where excessive concentrations may interfere with normal cellular functions or induce mild stress responses that suppress motility[41].

In conclusion, alfalfa honey significantly promotes keratinocyte migration at low concentrations, supporting its potential application as a natural agent in wound healing therapies. This effect is likely mediated by its rich polyphenolic content, which acts synergistically to enhance cellular responses essential for tissue regeneration.

3.7 Inhibition of Nitric Oxide Production in LPS-stimulated Macrophages

The anti-inflammatory activity of Palestinian alfalfa honey was assessed by measuring Nitric Oxide (NO) production in LPS-stimulated THP-1-derived macrophages. As shown in Figure 4, exposure to LPS elevated NO levels significantly to 370 ± 50% relative to the unstimulated control (100%), confirming successful macrophage activation. Treatment with alfalfa honey substantially reduced NO production to 110 ± 15%, indicating a marked anti-inflammatory effect.

This suppression of NO is strongly correlated with the honey's rich and diverse polyphenolic content. Quantitative analysis revealed a total polyphenol concentration of 47.37 mg/g, with notably high levels of ellagic acid (23.51 mg/g), *p*-coumaric acid (6.02 mg/g), kaempferol (4.00 mg/g), pinocembrin (6.3 mg/g), and myricetin (7.99 mg/g). These polyphenols are known to interfere with key inflammatory pathways, particularly by downregulating inducible nitric oxide synthase (iNOS) expression and inhibiting the NF-κB signaling cascade, which is essential for pro-inflammatory gene transcription[46-48].

Ellagic acid, for example, has been shown to inhibit NO release and pro-inflammatory cytokines in LPS-stimulated macrophages by targeting NF-κB and MAPK pathways[49,50]. Similarly, caffeic acid and pinocembrin have been documented to reduce iNOS expression and suppress oxidative burst in immune cells, mitigating the inflammatory response[37,51,52]. Flavonols such as kaempferol and quercetin also exhibit potent antioxidant activity, scavenging reactive oxygen species (ROS) and indirectly inhibiting NO synthesis[53,54].

The synergy among these polyphenols may amplify their bioactivity, contributing to the observed reduction

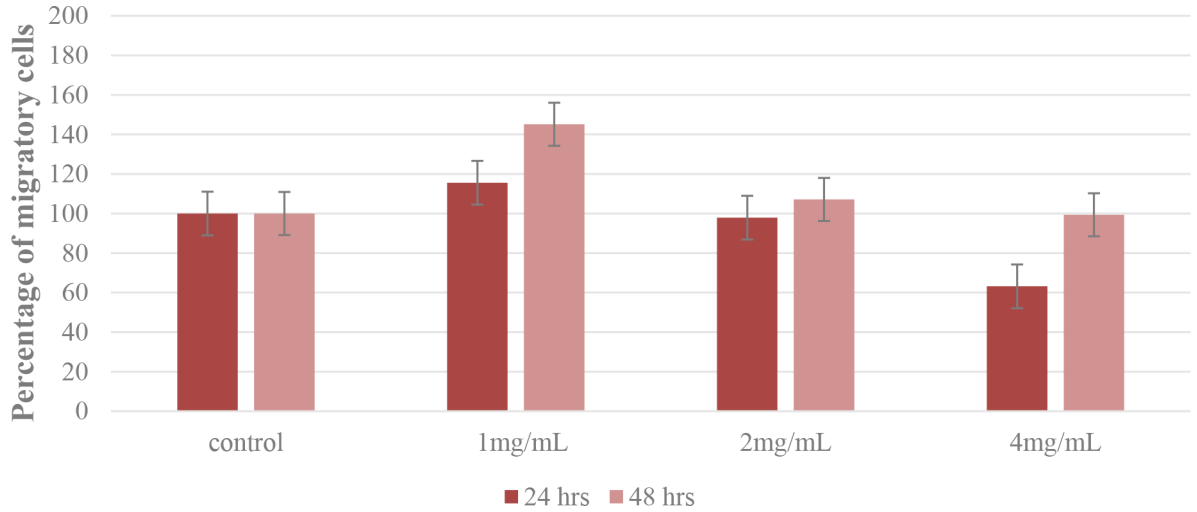

Figure 3. Alfalfa honey promotes HaCaT cell migration. Wound closure (%) was measured at 24 and 48 h post-treatment, normalised to untreated controls (100%). Data are mean ± SD of three independent experiments in triplicate.

in NO levels. The relatively high concentration of ellagic acid suggests it may play a dominant role in this activity, supported by secondary effects from pinobanksin, pinocembrin, and *p*-coumaric acid. Furthermore, the minimal cytotoxicity of the honey ensures that NO suppression is not due to general cell toxicity, but rather due to specific immunomodulatory mechanisms.

In conclusion, the observed inhibition of NO production in LPS-stimulated macrophages can be attributed to the high polyphenolic content of Palestinian alfalfa honey, particularly phenolics with well-documented anti-inflammatory properties. These findings support its potential role as a natural therapeutic agent for modulating macrophage-mediated inflammation and promoting wound healing.

3.8 Antibacterial Activity of Alfalfa Honey

The antibacterial properties of Palestinian alfalfa honey were assessed against a panel of Gram-positive and Gram-negative bacterial strains using Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays (Table 5). Among Gram-positive bacteria, *B. subtilis* showed the highest sensitivity, with both MIC and MBC values of 3.125% (w/w). *S. pneumoniae* displayed moderate sensitivity, with MIC and MBC values of 6.25% (w/w). For Gram-negative bacteria, *K. pneumoniae* was the most susceptible, with MIC and MBC values of 3.125% and 6.25% (w/w), respectively. *H. influenzae* was comparatively less sensitive, showing MIC and MBC values of 12.5% and 25% (w/w), respectively.

These findings confirm that alfalfa honey exhibits broad-spectrum antibacterial activity, in agreement with earlier studies demonstrating honey's effectiveness against both Gram-positive and Gram-negative pathogens, including multi-drug resistant (MDR) strains such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*[55,56]. The antimicrobial activity of honey is attributed to multiple factors, including low pH, high sugar content leading to osmotic stress, enzymatically generated hydrogen peroxide (H_2O_2), and the presence of bioactive compounds such as phenolics and antimicrobial peptides[56].

Notably, honey's efficacy against MDR bacteria has been documented in several studies. Manuka honey, for example, has shown the ability to restore bacterial susceptibility when used in combination with antibiotics, a mechanism that may help in reducing

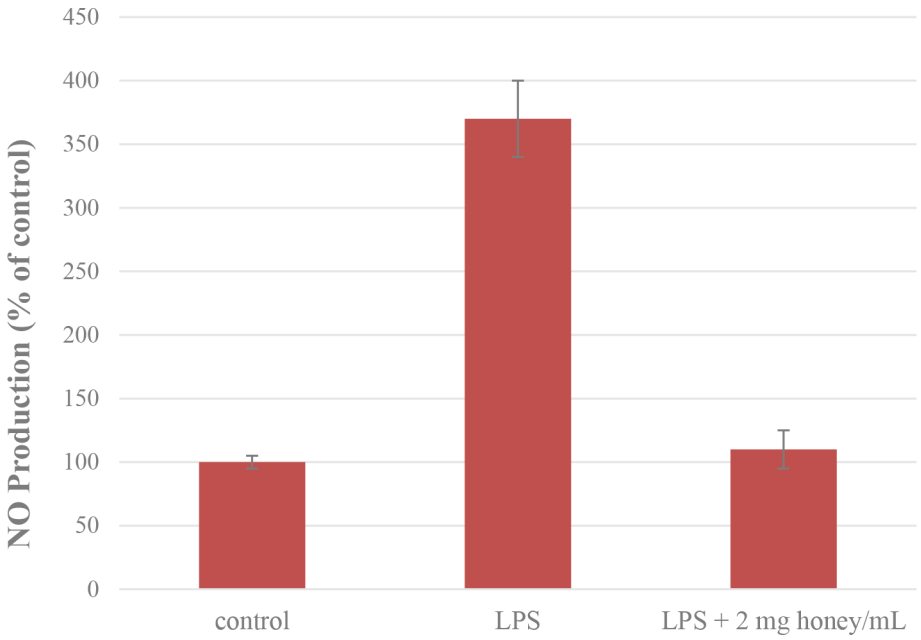

Figure 4. Anti-inflammatory effect of alfalfa honey in LPS-stimulated macrophages. Alfalfa honey attenuates LPS-induced inflammation in THP-1-derived macrophages. Cells were co-treated with Lipopolysaccharide (LPS) and alfalfa honey (2 mg/mL) for 72 h. Inflammatory response was quantified and expressed as a percentage relative to the untreated control (set at 100%). Data represent the mean ± Standard Deviation (SD) of three independent experiments.

Table 5. Antibacterial activity of alfalfa honey. Antibacterial activity of alfalfa honey against various bacterial strains (MIC and MBC values in W/W %)

Gram-positve				Gram-negative			
S. pneumoniae		*B. subtilis*		*H. influenzae*		*K. pneumoniae*	
MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
6.25	6.25	3.125	3.125	12.5	25	3.125	6.25

antibiotic resistance[57-61]. This synergistic property further underscores honey's clinical potential in treating infected wounds.

Another critical factor in chronic wound infections is biofilm formation, which occurs in up to 80% of such cases[62]. Honey has been shown to disrupt biofilms and inhibit their formation, thereby enhancing healing and preventing recurrence[62-64]. Although our current study did not evaluate antibiofilm activity, the observed bactericidal effects of alfalfa honey suggest promising future applications in this area.

It is important to highlight that the antimicrobial efficacy of honey varies depending on its botanical source and chemical composition. For instance, Medihoney, derived from Manuka honey, contains high levels of Methylglyoxal (MGO), which is responsible for its non-peroxide antibacterial action. In contrast, Revamil honey relies on H_2O_2 production and bee defensin-1, demonstrating a peroxide-dependent mechanism[56]. Interestingly, MGO can degrade key enzymatic proteins like glucose oxidase and bee defensin-1, suggesting that both mechanisms may not coexist effectively in the same honey type[65,66].

In this context, the potent antibacterial activity observed for alfalfa honey, particularly against *B. subtilis* and *K. pneumoniae*, suggests the presence of active antimicrobial compounds and possibly peroxide-dependent mechanisms. Further phytochemical characterisation is needed to elucidate the exact compounds responsible for this activity and their stability under physiological conditions.

4. Conclusions

The present study provides a comprehensive characterisation of Palestinian *M. sativa* (alfalfa) honey and demonstrates its therapeutic potential in wound management. Detailed physicochemical profiling revealed favorable attributes, including low moisture content, acidic pH, enriched protein levels, and balanced mineral composition, which collectively create an environment that supports the stability and bioactivity of its natural constituents. High-performance liquid chromatography confirmed a diverse polyphenolic composition dominated by ellagic acid, myricetin, and pinocembrin, compounds known for their strong antioxidant and anti-inflammatory activities.

Biological assays demonstrated that alfalfa honey exerts potent antioxidant effects, reduces nitric oxide production in LPS-stimulated macrophages, and promotes keratinocyte proliferation and migration at appropriate concentrations. These findings suggest that the honey's polyphenolic compounds may contribute to cellular protection against oxidative stress and modulation of inflammatory pathways, thereby facilitating tissue repair. In addition, broad-spectrum antibacterial activity was observed against both Gram-positive and Gram-negative strains, with the data suggesting that multiple factors—including acidity, osmotic effect, enzymatic hydrogen peroxide release, and phenolic compounds—act together to inhibit bacterial growth. Although these interactions are promising, further mechanistic studies are required to confirm the exact pathways involved.

Taken together, the findings validate the traditional use of alfalfa honey as a natural remedy for wound healing and support its development as a bioactive therapeutic substance. Nevertheless, *in vitro* results represent only an initial step, and translation to clinical application will require *in vivo* animal models and controlled clinical trials. Future studies should also investigate the molecular targets of key polyphenols, the dose-dependent effects on different skin cell populations, and potential synergistic interactions with conventional wound-care treatments. Moreover, formulating alfalfa honey into advanced delivery systems—such as hydrogels, ointments, or biopolymer-based dressings—could enhance its bioavailability, stability, and usability in medical settings. Comparative studies across honeys of different botanical and geographical origins will be critical to establish standardised benchmarks for efficacy, safety, and therapeutic claims.

In conclusion, Palestinian *M. sativa* honey represents a rich natural source of bioactive compounds

with antioxidant, anti-inflammatory, antibacterial, and wound-healing properties. By combining traditional knowledge with modern experimental validation, this study contributes to the growing scientific evidence supporting the clinical potential of honey in wound management and highlights the need for further translational research to fully harness its healing capacity.

5. Author Contribution

Methodology, writing - review and editing, review and editing: [DJ, MM, DW], Formal Analysis (AK lead), writing - review and editing (equal): [AK, ST], Conceptualisation (supporting); Writing - original draft (supporting); Writing - review and editing (equal). [BL, BS]. All authors have read and agreed to the published version of the manuscript.

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