Arab American University
Faculty of Graduate Studies
Department of Health Sciences
Master Program in Cellular and Molecular Bio Sciences

In Vitro Evaluation of Wound Healing Effects of Three Palestinian Honey Types Using Cells from the Human Keratinocyte Cell Line

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This Thesis Was Submitted in Partial Fulfilment of the Requirements for the Master Degree in Cellular and Molecular Bio Sciences.

Palestine, Feb /2026

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Thesis Approval

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Declaration

I declare that, except where explicit reference is made to the contribution of others, this thesis is substantially my own work and has not been submitted for any other degree at the Arab American University or any other institution.

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Dedication

I dedicate this thesis to my beloved parents, whose unwavering support, prayers, and encouragement have been my constant source of strength throughout this journey. I am deeply grateful to my family and friends for their endless support and companionship. Finally, I sincerely thank my supervisors for their guidance, advice, and invaluable mentorship.

Student Name: Donia Ahmad Taher Jarrar

Acknowledgment

I dedicate this work to the courageous souls who endure imprisonment and hardship with unwavering resolve, inspiring all of us to persist in the pursuit of justice and freedom. I also honor the martyrs who gave their lives for Palestine, whose sacrifice continues to light the path of hope and resilience for generations to come.

I would like to sincerely thank my distinguished supervisor, Prof. Bashar Saad, for his invaluable guidance, advice, and support throughout this research project. I am also grateful to the research assistant, Doha Waldali, for her assistance and dedication, which greatly facilitated the progress of this study.

I am profoundly grateful to my mother, whose love, encouragement, and guidance have been the cornerstone of my journey, and to my father, whose principles and example continue to guide me. I also extend my heartfelt thanks to my siblings for their constant support, patience, and unwavering presence throughout this journey.

I also extend my sincere thanks to the research team whose scientific input and collaboration significantly enhanced the quality of this work. In particular, I would like to acknowledge Dr. Hamada Imtara from the Faculty of Medicine, as well as Dr. Basheer Abu-Farich and Dr. Mahmoud Masalha at the Al-Qasemi Research Center, for their cooperation, expertise, and professional support.

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Abstract

Wound healing is a complex biological process that requires coordinated cell activities such as keratinocyte proliferation, migration, regulation of inflammation, and antimicrobial protection. Honey has been traditionally utilized for its use in the treatment of wounds. However, there has been a lack of scientific data available for comparison of the biological activities of different honeys. In this study, we examine the use of the following Palestinian honeys: avocado honey, alfalfa honey, and citrus honey.

The study employed HaCaT cells to determine cell viability, proliferation, and migration and re-epithelialization capabilities by scratch assays, while the inflammatory response was tested in THP-1 cells by measuring nitric oxide production by Griess assays. Furthermore, the antibacterial properties of each honey type against pathogens found in wounds were analyzed. Molecular docking studies were also performed to determine possible interactions between bioactive compounds found in honey and major molecular targets that take part in wound healing.

The results clearly show that all honey samples had positive and unique effects on wound healing processes. The enhancement of wound closure and cell migration for avocado and alfalfa honeys surpassed other honeys like citrus honey; hence, it indicated a higher involvement of the honey samples in the wound healing process of re-epithelialization. The honey samples have been shown to have some levels of anti-inflammatory and antibacterial properties, indicating that the presence of chemicals with varied levels makes up the basic difference in the performance of the honey samples. The docking study affirmed this by indicating positive interactions of the honey compounds with proteins involved in processes like cell migration and inflammation pathways.

The potential of Palestinian honey to be an alternative or a supplement to modern wound treatment is brought to the limelight by this piece of research. It is an affirmation of their long-unrefuted use by supporting their efficacy in an in-vivo study.

Keywords: Wound healing, Palestinian honey, Keratinocytes, Inflammation, Re-epithelialization

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List of Definitions of Abbreviations

- 5-LOX: 5-Lipoxygenase
- AAE: Ascorbic Acid Equivalent
- AChE: Acetylcholinesterase
- AMPK: AMP-Activated Protein Kinase
- ATCC: American Type Culture Collection
- aw: Water Activity
- Be: Binding energy ($kcal \cdot mol^{-1}$)
- bFGF: Basic Fibroblast Growth Factor
- BChE: Butyrylcholinesterase
- CFU: Colony Forming Unit
- CLSI: Clinical and Laboratory Standards Institute
- CO_2: Carbon Dioxide
- DMSO: Dimethyl Sulfoxide
- DHA: Dihydroxyacetone
- DHFR: Dihydrofolate Reductase
- DMEM: Dulbecco’s Modified Eagle Medium
- DPPH: 2,2-Diphenyl-1-picrylhydrazyl
- ECM: Extracellular Matrix
- EGF: Epidermal Growth Factor
- EGFR: Epidermal Growth Factor Receptor
- EMT: Epithelial–Mesenchymal Transition
- ERK: Extracellular Signal-Regulated Kinase
- ERK1/2: Extracellular Signal-Regulated Kinases 1 and 2
- FBS: Fetal Bovine Serum
- FRAP: Ferric Reducing Antioxidant Power
- GLP: Good Laboratory Practice
- H_2O_2: Hydrogen Peroxide
- HaCaT: Human Adult Low-Calcium High-Temperature Keratinocyte Cell Line
- HEP: Honey Extract Polyphenols

- IC_{50}: Half Maximal Inhibitory Concentration
- Ic: Inhibitory Concentration (μM)
- iNOS: Inducible Nitric Oxide Synthase
- IL: Interleukin
- IL-1: Interleukin-1
- IL-1β: Interleukin-1 Beta
- IL-6: Interleukin-6
- IL-8: Interleukin-8
- IL-10: Interleukin-10
- IRB: Institutional Review Board
- KGF: Keratinocyte Growth Factor
- LGA: Lamarckian Genetic Algorithm
- LDH: Lactate Dehydrogenase
- LPS: Lipopolysaccharide
- MAPK: Mitogen-Activated Protein Kinase
- MBC: Minimum Bactericidal Concentration
- MGO: Methylglyoxal
- MIC: Minimum Inhibitory Concentration
- MHn: Manuka Honey
- MLC: Minimum Lethal Concentration
- MMPs: Matrix Metalloproteinases
- MM6: MonoMac-6 Cell Line
- MTT Assay: A colorimetric assay used to assess cell viability and proliferation based on mitochondrial activity
- NAD: Nicotinamide Adenine Dinucleotide
- NED: N-(1-Naphthyl) Ethylenediamine Dihydrochloride
- NETs: Neutrophil Extracellular Traps
- NF-κB: Nuclear Factor Kappa B
- Nrf2: Nuclear Factor Erythroid 2–Related Factor 2
- NO: Nitric Oxide

- PBS: Phosphate-Buffered Saline
- PDGF: Platelet-Derived Growth Factor
- PGC-1α: Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha
- PMA: Phorbol 12-Myristate 13-Acetate
- PDB: Protein Data Bank
- RP: Reducing Power
- ROS: Reactive Oxygen Species
- RPMI-1640: Roswell Park Memorial Institute culture medium
- SD: Standard Deviation
- SIRT1: Sirtuin 1
- THP-1: Human monocytic leukemia cell line
- TIMPs: Tissue Inhibitors of Metalloproteinases
- TFC: Total Flavonoid Content
- TGF-β: Transforming Growth Factor Beta
- TLR: Toll-Like Receptor
- TNF-α: Tumor Necrosis Factor Alpha
- TPC: Total Phenolic Content
- UMF: Unique Manuka Factor
- VEGF: Vascular Endothelial Growth Factor
- vWF: von Willebrand Factor
- w/w%: Weight per weight percent

1 Chapter One: Introduction

1.1 Study Background

Wound healing is a crucial physiological process that maintains the viability of an organism by restoring the tissue structure and function after injury (Mamun A. , Shao, Geng, Wang, & Xiao, 2024). It initiates a cascade of multiple systems (including the vascular, immune, and integumentary systems) that close the defect. Besides preserving blood integrity and preventing fluid loss, it also protects the tissue from infection by closing the wound site, forming a new barrier, and activating immune defenses that prevent microbial invasion (LaPelusa & Dave, 2019). The successful healing of a wound is vital to the integrity of the skin. Therefore, understanding the wound healing process and what regulates it is a key priority in medical and biological research.

Since the wound healing disruption results in serious damage beyond the loss of tissue, it's important to understand the wound healing process. Globally, tens of millions of people suffer from impaired or delayed wound healing caused by diabetes, vascular disease, or aging. These conditions often lead to chronic wounds that are persistent, disabling, and difficult to treat (Sen, 2023).

The impact of chronic wounds extends beyond the individual to the healthcare system. Compromised healing wounds have an enormous economic cost to health care systems via extended care, hospitalization, and extremely high financial costs. Other than the economic factor, chronic wounds also have a severe effect on patients' social functioning and mental well-being.

Traditional therapies (including debridement, dressings, negative pressure wound therapy, and systemic or topical antibiotics) may have helped wound closure and infection prevention rates increase, but are not successful in the management of complex or chronic wounds. The limitations of traditional therapies include high recurrence rates, risk of

antibiotic resistance, and poor efficacy in the treatment of underlying etiologies such as diabetes, vascular insufficiency, or age-related dysfunction.

As a result, there is an urgent need for therapeutic interventions that not only facilitate healing but also maximize tissue repair quality overall. This has stimulated scientific research into complementary and natural therapies, including plant extracts, aloe vera, and honey, which have shown encouraging results in wound management. Among these natural agents, honey holds particular promise for wound treatment due to its broad antimicrobial activity, ability to modulate inflammation, and capacity to stimulate tissue regeneration, which have been increasingly investigated for their therapeutic applications in advanced wound healing.

1.1.1 Biology of Wound Healing

Wound healing initiates at the moment of tissue damage and is divided in the classical setting into four phases that overlap one another: hemostasis, inflammation, proliferation, and remodeling (Gurtner, Werner, Barrandon, & Longaker, 2008), as shown in Figure 1.1

The wound healing process starts with Hemostasis, which occurs within seconds of injury, with platelet activation and vasoconstriction forming a fibrin plug that seals off the wound and prevents further bleeding. Platelets also release bioactive molecules, such as growth factors, that trigger subsequent events (Fernández-Guarino, Hernández-Bule, & Bacci, 2023).This is followed by the Inflammation phase subsequently ensues as immune cells infiltrate the tissue to eliminate invading pathogens and trash. Neutrophils and macrophages are key, secreting cytokines and chemokines to modulate the early healing process (Mamun A. , Shao, Geng, Wang, & Xiao, 2024) .

The Proliferation follows the inflammation phase, which is marked by migration and division of endothelial cells, fibroblasts, and keratinocytes. Fibroblasts lay down the components of the extracellular matrix, such as collagen, and keratinocytes move to re-cover the wound surface. New vessels are established to nourish the regenerating tissue (Kamysz & Kleczkowska, 2025). Finally, the Remodeling (Maturation) phase, where collagen is

remodeled, and tensile strength is gained. However, the healed tissue barely acquires the original properties of uninjured skin (Mamun A. , Shao, Geng, Wang, & Xiao, 2024).

Even though these processes are tightly regulated, factors such as diabetes, infection, vascular insufficiency, or aging can interfere, leading to impaired or delayed wound healing with the resulting occurrence of chronic wounds or over-scarring.

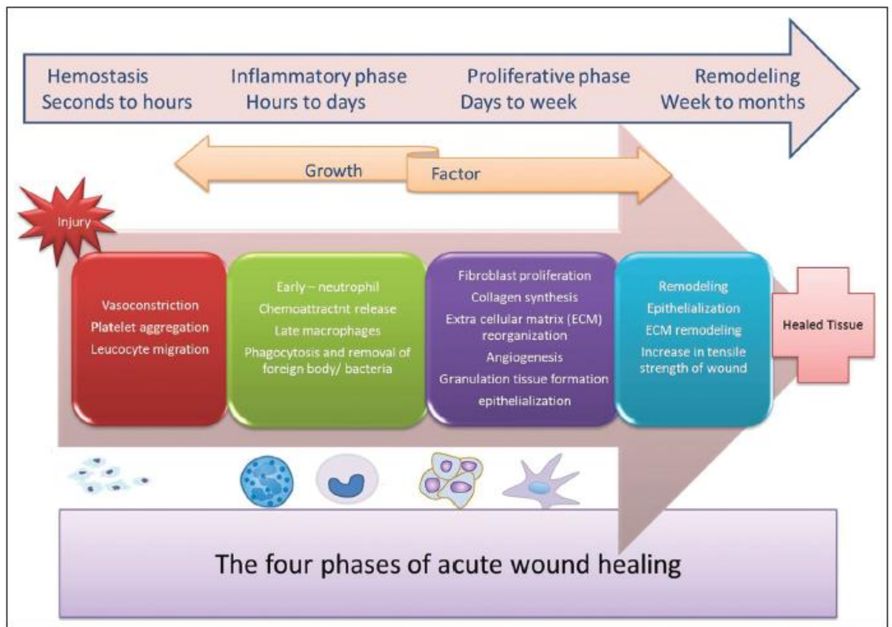

Figure 1.1: The four phases of normal wound healing

1.1.2 Acute and Chronic Wounds

Wounds normally are grouped into two categories, which are acute and chronic, based on their healing behavior:

- Acute wounds such as trauma injury, burns, or surgical injury. They will progress through the routine repair cascade and heal within a predictable time span.

- Chronic wounds, however, do not follow the normal healing process. They remain in chronic inflammation, never advancing into tissue regeneration.

Chronic wounds are becoming a growing global problem. They affect 1–2% of the population in the developed world, with expected increases in prevalence as a result of aging populations and rising levels of diabetes, obesity, and cardiovascular disease (Maheshwari, 2024). In the United States alone, chronic wound care in healthcare costs over $25 billion annually (Nussbaum, et al., 2018).

These are further complicated by infection by commonly antibiotic-resistant agents like Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa. They contribute to healing delay, costliness, and adverse patient outcomes (Wangoye, Mwesigye, Tungotyo, & Twinomujuni, 2022). Understanding the molecular mechanisms underlying impaired healing and exploring novel interventions, such as thymoquinone from Nigella sativa, may help accelerate wound closure and improve recovery (Kmail, Said, & Saad, 2023).

1.1.3 Limitation of Human Healing Capacity

Human tissue repair is suboptimal even under ideal conditions. Healed tissue has decreased function relative to normal tissue and possesses only 70–80% normal skin tensile strength (Khalid, Nawi, Zulkifli, Barkat, & Hadi, 2022). Even if other animals, such as salamanders and planarians, can regenerate entire structures, adult human beings have very little regenerative capability. Complete regeneration is comparatively restricted to fetal life, in which case, wound healing occurs without scarring. Scarring is inevitable in adults due to the partial reconstruction of the original tissue architecture.

These biological limitations are what emphasize the need for therapeutic techniques not only to improve closure, but to improve the quality of the repair as well.

1.1.4 Issues in Wound Care Today

Wound care has also improved with new technologies such as skin grafting, bioengineered dressings, and negative pressure wound therapy (Kondej, et al., 2024).

However, despite these, many significant issues remain. Such as Antibiotic Resistance , Increased antimicrobial resistance decreases the efficacy of traditional infection control methods (Monk, et al., 2024). And the treatment of chronic wounds is time-consuming, labor-intensive, and costly to patients and healthcare organizations (Sen, 2025). Additionally, Contemporary therapies are aimed at closure more than toward the healing function of the tissue, leading to recurrence or chronic functional deficit (Sharma, Sharma, & Zhao, 2023).These considerations have generated new interest in complementary and natural therapies that are efficacious, safe, and cost-effective.

1.1.5 Honey as a Therapeutic Agent

Honey has always been employed as a medicinal agent in wound care for thousands of years, substantiated by evidence from ancient Greece and Egypt and traditional medicine globally. In the modern world, the evidence from science attests to its broad-spectrum therapeutic value (Saikaly & Khachemoune, 2017). Honey exhibits Antibacterial Activity due to its acidity, high sugar content, production of hydrogen peroxide, and some bioactive compounds such as methylglyoxal in Manuka honey, making it effective against a wide range of bacteria, including MRSA and Pseudomonas aeruginosa (Ogwu & Izah, 2025). Honey also has Anti-inflammatory Activity by inhibiting inflammation and oxidative stress by suppressing pro-inflammatory cytokines (Iosageanu A. , Stefan, Craciunescu, & Cimpean, 2024).

In addition, honey has a Moist Healing Environment, which creates a healing-friendly environment by imparting sufficient moisture and preventing scarring (Zainuddin, et al., 2025).it also enhances tissue repair by stimulating fibroblast and keratinocyte activity to allow quick closure and re-epithelialization (Gościniak, Attard, Malesza, Kamiński, & Cielecka-Piontek, 2025)

These synergistic actions have led to the use of honey in a variety of modern wound care products, including medical-grade honey dressings. These effects are shown in Figure 1.2 which illustrates the influence of honey on the phases of wound healing. During the hemostasis, honey stabilizes the wound bed by reducing pH and oxidative cell damage and

limiting microbial burden. During the inflammatory phase, honey generates hydrogen peroxide, modulates pro-inflammatory cytokines, and has an antioxidant effect, controlling infection and inflammation.

In the proliferative phase, honey promotes epithelialization, granulation tissue, reduction of wound edema, and exudate to enable tissue repair. In the remodeling phase, honey promotes the remodeling of wounds, repair of tissues, and reduction of scar formation. In general, the figure demonstrates that honey's antibacterial, anti-inflammatory, and tissue-regenerative properties have a synergistic effect during the process of wound healing.

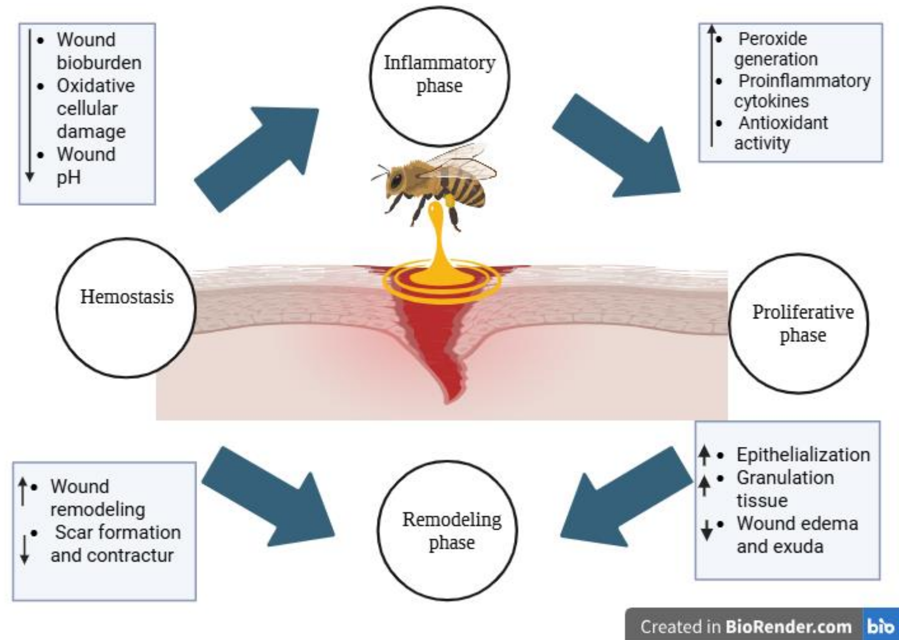

Figure 1.2: Honey's therapeutic effects on the wound healing process

1.1.6 Cellular Mechanisms and Knowledge Gaps

Keratinocytes are the most numerous cell type of the epidermis, the outermost layer of the skin, and are the main cells in skin barrier maintenance. During wound healing, keratinocytes migrate into the wound bed, proliferate to close the wounded area, and differentiate to restore the protective barrier (Muñoz, Vásquez, & del Sol, 2020). Honey promotes these processes by enabling keratinocytes migration, getting closer to the wound more quickly. Also, Keratinocytes proliferation, inducing vigorous re-epithelialization. This

result of honey's high density of sugars, amino acids, and bioactive phenolic compounds that provide energy and activate signaling pathways like MAPK/ERK, which stimulate cell proliferation (Wani, et al., 2020).

In addition, Inflammation regulation is one of the most important effects of, which prevents chronic inflammatory response that harms healing. through antioxidant activity and modulation of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, maintaining a balanced environment conducive to tissue repair (Tashkandi, 2021). Collectively, these multifaceted effects make honey an effective natural agent in supporting keratinocyte-mediated wound closure.

While such activities are noted for some honeys, particularly the Manuka honey, little comparative data exist on other honey types (Barazesh P. , et al., 2025). There is Multiples of honeys with different chemical compounds with high therapeutic value still being researched due to the diversity of floral resources found throughout the world.

1.1.7 Palestinian Honeys: An Untapped Resource

Palestine's distinctive flora is naturally suited to producing a range of honeys, including avocado, alfalfa, and citrus honeys (Abu-Farich & et al., 2024). They are highly valued locally for their powerful antimicrobial effect, but there is little scientific evidence for their medical value. Investigating theses honeys can Provide scientific backing for traditional medicine and improve global wound care solutions through the provision of new, natural treatments. Moreover, it can Make sustainable apiculture and local economic development possible.

1.2 Problem Statement and Research Question

Wound healing is a critical process for restoring skin integrity following injury. A wide variety of wounds, especially chronic ones, fail to heal properly delaying the phases of the

wound healing stages, leading to patient suffering, increased healthcare costs, and heightened risk of infection. Despite the huge advancements in wound care technologies, the current treatments often fall short due to several complications, such as antimicrobial resistance and limited tissue regeneration capacity. This underscores the importance of studies that address natural treatments such as honey.

There is limited comparative research on how different types of honey affect wound healing processes. Specifically, the distinct biological effects of avocado, alfalfa, and citrus honeys on human keratinocytes, the primary cells involved in re-epithelialization, remain underexplored, particularly in controlled in vitro environments, despite their effectiveness having been proven in clinical treatment.

Demonstrating that these honeys can enhance wound healing will help develop effective and alternative wound healing treatments. The types of Palestinian honeys, such as avocado, alfalfa, and citrus, are not covered in a comparative study despite their strong antimicrobial effects, which play a vital role in determining their overall therapeutic value.

The main hypothesis of my thesis is that all selected types of honey (avocado, alfalfa, and citrus) will have a positive effect in the wound healing process, each to a different degree. This study will address these questions:

1. How does citrus, alfalfa, and avocado honey affect the viability and proliferation of HaCaT keratinocytes?
2. Are there significant differences in keratinocyte viability and proliferation between the three types of honey
3. How does citrus, alfalfa, and avocado honey affect the rate of wound closure in the scratch test?
4. Which honey type most effectively promotes in vitro wound closure?

5. How do citrus, alfalfa, avocado, and honey affect inflammation in THP-1 cells as measured by the Griess nitric oxide assay?
6. Which honey type demonstrates the greatest anti-inflammatory effect as measured by the Griess assay?
7. Are there significant differences in the effectiveness of wound healing between the different types of honey tested
8. What is the antibacterial activity of each honey type against relevant wound pathogens?
9. What are the potential molecular interactions of honey bioactive compounds with key wound healing targets, as assessed by docking experiments?

1.3 Objectives of the Study

The study aims to evaluate the healing advantages and ability that those honey types can provide, which may contribute to the development of personalized wound care strategies that enhance recovery, which might also function as an opportunity.

Secondly, this study fills massive gaps in the current literature regarding avocado, alfalfa, and citrus honeys that haven't been investigated in laboratory settings, such as in vitro skin cells that mimic wounds, even though their efficiency has been validated in other clinical researches.

Thirdly, this research will help to expand our knowledge of the wound recuperation complex procedure on the cellular and molecular stages; additionally, it's going to add to our knowledge of the role the honey plays in the wound healing. This study will provide valuable insights into the comparative effects of different types of honey on wound healing, which may lead to the development of more effective therapeutic agents. Clinical applications help to gain a deeper understanding of the wound healing process by:

1) Evaluation of the effects of avocado, alfalfa, citrus, and Manuka honey on the proliferation of HaCaT keratinocytes.

2) Evaluate the effect of these types of honey on the re-epithelialization process.
3) Evaluate the effect of these types of honey on keratinocyte migration.
4) Evaluate the effect of these types of honey on the wound inflammation process.
5) Evaluate the antibacterial activity of each honey type against relevant wound pathogens.
6) Investigate potential molecular interactions of honey bioactive compounds with key wound healing targets through docking experiments.

1.4 Significance of the Study

This study investigates the comparative wound-healing properties of avocado, alfalfa, and citrus honeys, which are poorly researched types relative to highly researched ones. By focusing on these unsophisticated honeys in a controlled in vitro system, the study addresses a clearly delineated knowledge gap and provides evidence that may validate their traditional use. Identification of the biological mechanism underlying their healing effects is critical in integrating them into current, evidence-based strategies for wound care.

The importance of this research lies in its contribution to the creation of low-cost and effective therapeutic options. Chronic wounds are a growing health problem worldwide, especially in resource-limited communities. The success of applying local honey types in this research can trigger their use as inexpensive substitutes or supplements to current treatments. This would not only improve patient well-being but also make locally made natural products sustainable for use.

Additionally, this research opens new doors for future investigations into the molecular mechanisms of honey-induced wound healing, such as cytokine regulation,

extracellular matrix remodeling, and oxidative stress management. These study's results may inspire and encourage the creation of advanced honey-based products, such as bioactive dressings or hydrogel formulations. By combining traditional treatment with evidence-led science, the work contributes to international science and domestic health innovation.

1.5 Thesis Organization

This thesis has been separated into distinct sections to guarantee a well-organized, consistent flow of ideas and, most importantly, in accordance with the standards for thesis writing of the Arab American University. Every part of the study, from the basic concepts to the analysis, application, and results, focuses on a different aspect. While providing a comprehensive understanding of the research problem, the framework aims to highlight significant findings and practical insights. It is as follows:

- Chapter I: Introduction

  The chapter offers an introduction to the biological significance of wound healing and the traditional and emerging uses of honey in therapy. The chapter is utilized to state the problem of research, outline the objectives and questions, and present the significance of exploring the Palestinian honey types in wound healing. This chapter also provides an introduction to the thesis.

- Chapter II: Literature Review

  This chapter outlines the fundamental biology of the healing process phases, wound classification, and factors influencing this process. It then describes the bioactive activity of honey, the molecular mechanisms, and the role of keratinocytes in wound healing. A comprehensive assessment of Citrus, Alfalfa, Avocado, and Manuka honeys is presented, based on their chemical composition, clinical and in vitro information, which will provide a solid stand of the importance of this research, and finally, the current research gaps. The chapter concludes with a comparative summary of the reviewed honeys.

- Chapter III: Methodology

  Experimental procedure and design of the study are discussed in this chapter. preparation of the studied honey samples, the cell culture procedure for each HaCaT and THP-1 cell line, and the assays used (MTT for cytotoxicity, scratch assay for migration, and Griess assay for inflammation) are described. Analytical and statistical methods used to analyze the data, along with ethical concerns, are provided.

- ➢ Chapter IV: Results

  This chapter presents the findings of the study in quantitative cytotoxicity, migration, and nitric oxide data. Statistical evaluations, tables, and graphs represent the effect of each category of honey on keratinocyte viability, percentage migration, and inflammation. Observations are divided into the experimental group and the type of assay.

- Chapter V: Discussion

  This chapter is a presentation of the findings based on the literature. It compares the biological activity of Citrus, Alfalfa, and Avocado honeys and addresses how the work contributes to current literature on natural wound healing products. Points of incompatibility with previous research, study limitations, and potential biological mechanisms are addressed.

- Chapter VI: Conclusion and Recommendations

  This chapter provides a summary of the main findings and results in wound healing research and natural therapy development. Future directions for research are suggested, such as conducting more in vitro, in vivo, and clinical trials on Palestinian honeys.

- Chapter VII: References

This last chapter includes all academic sources, journals, books, and databases referred to throughout the thesis, presented in the citation style recommended by the university.

2 Chapter Two: Literature Review

This chapter offers a detailed examination of previous studies related to this research. Starting with the fundamentals of wound healing biology, including the distinctive phases of wound healing, classification of wounds into acute or chronic wounds, and determinants of healing efficiency. These are the essentials for understanding the complex processes required to restore tissue integrity and the barriers that often contribute to poor or delayed healing. Its role as a primary mediator of re-epithelialization is further highlighted, as its activity forms the main aim of this study.

Following this, the chapter will dive into the therapeutic uses of honey as an organic wound-healing substance with a specific focus on its cultural and medicinal significance in Palestine. It elaborates on honey's beneficial bioactive properties, including its antibacterial, antioxidant, anti-inflammatory, and immunomodulatory activities, as well as its ability to stimulate cell proliferation, angiogenesis, and extracellular matrix deposition. Molecular mechanisms under honey's control are discussed, too, supported by in vitro and in vivo evidence demonstrating its effect on keratinocyte function and immune responses.

Concludes by comparably evaluating honeys examined in this current study, Citrus, Alfalfa, and Avocado, and their origin, composition, and biological activities, comparatively with Manuka honey as a clinically proven one serving as a positive control. By integrating recent information and addressing existing literature gaps, this review fills the need to compare these less-investigated honeys under a controlled laboratory setting. It provides the scientific basis for the experimental design presented in the following chapter and emphasizes the relevance of this work towards the establishment of natural wound care approaches

2.1 Wound Healing

Wound healing is a complex process that involves a cascade of controlled biological events that occur in a coordinated manner to ensure the recovery of damaged tissue resulting from injury. Regardless of whether the injury results from trauma, surgery, or illness, tissue destruction results in the loss of organ and tissue function, which triggers the initiation of a cascade of steps to restore integrity and homeostasis in the injured tissues (Mamun A. , Shao, Geng, Wang, & Xiao, 2024).

2.1.1 Phases of Wound Healing

Many models have defined the process of wound healing. Earlier studies often presented a simplified view of the dynamics of healing through the three-phase model, which consists of the phases of inflammation, proliferation, and remodeling. This model was largely based on broad observable tissue-level events and provided only an overall outline of the healing sequence. An approach such as the two-phase model, separating healing into two phases, inflammatory and regenerative, offers another way to conceptualize the process. Investigators proposed this distinction to highlight the major functional transition from damage control to tissue rebuilding, rather than detailing individual cellular processes. Particularly regarding chronic wounds, the five-stage model is a more contemporary idea that divides particular actions for closer inspection. This model was developed in order to encapsulate further pathological characteristics that are associated with impaired healing, including sustained inflammation and deranged proliferation. (Eming, Martin, & Tomic-Canic, 2014).

However, the four-phase model is the most often accepted and thorough model available today. Since it is based on well-characterized cellular, molecular, and biochemical events, it presents the most detailed yet practical framework. In this research, the four-phase model of wound healing will represent the process, since it offers a better and more ordered framework for assessing the effects of therapeutic agents such as honey on the several phases of wound recovery, especially in terms of inflammation modulation, cellular proliferation, and re-epithelialization (Gurtner, Werner, Barrandon, & Longaker, 2008).

To understand the wound healing process according to the four-phase model, Figure 1.1 highlights the normal wound healing phases. The figure depicts how cell and molecular mechanisms are controlled for the best restoration of injured tissue.

Hemostasis Phase

The First phase and the urgent one of the wound healing stages, a localized reaction that is intended to begin the repair cascade and stop the blood loss from the injury. Its reaction typically takes from seconds to minutes, starting with vascular constriction, the first response for a reflexive vasoconstriction, which will narrow the blood vessels, resulting in minimizing the loss of blood (Cioce, Cavani, Cattani, & Scopelliti, 2024). This reaction is mediated by local nerve reflexes and the release of constrictive substances like endothelin from endothelial cells, platelet aggregation, after the palatal are exposed to subendothelial components as collagen. Von Willebrand factor (vWF) triggers a cascade of events (Liu, et al., 2025). The platelet will change shape, releasing granules that aggregate to form a temporary platelet plug at the injury site, which will cause platelet attachment to the extracellular matrix (ECM).

The coagulation cascade is activated via the intrinsic and extrinsic pathways, and fibrinogen is transformed into fibrin. creating a scaffold for cellular infiltration and a sturdy mechanical wall for the clot (Bayer, 2022). The fibrin clot acts as a provisional matrix for migrating cells and provides structural support for the early wound.

These migrating cells also secrete various growth factors, including transforming growth factor-beta (TGF-β), which regulates inflammation and stimulates fibroblast proliferation, and platelet-derived growth factor (PDGF), which attracts neutrophils and macrophages. This will help shift into the following stage of healing, the Inflammatory phase, and indicate the recruitment of immune cells (Laurens, Koolwijk, & de Maat, 2006).

Inflammatory Phase

The second phase, following the hemostasis, starts hours after the injury and lasts 48 to 96 hours following the injury. This phase is critical for clearing pathogens, cellular debris, and damaged tissue components, laying the foundation for the repair and regeneration process (Gao, et al., 2024). This is achieved by the activation of the immune response and the infiltration of the immune cells.

The process starts due to damaged cells, platelets, and the fibrin matrix from the hemostasis phase, releasing various pro-inflammatory cytokines and chemokines such as Interleukin-1 (IL-1), Tumor necrosis factor-alpha (TNF-α), and Interleukin-6 (IL-6). These mediators initiate and amplify the recruitment of the immune cells to the site of injury, ensuring a rapid and targeted response to tissue damage. The first white blood cells to reach the wound site are neutrophils. They peak within 24–48 hours, and are directed there by specific biological signals like complement factors and interleukin 8 (IL-8) (Diegelmann & Evans, 2004). Neutrophils carry out important tasks like phagocytosing pathogens, removing debris, releasing proteases and reactive oxygen species (ROS) and proteolytic enzymes like elastase and matrix metalloproteinases (MMPs) to break down damaged tissue. Finally, they form neutrophil extracellular traps (NETs) to trap and neutralize microbes (Gao, et al., 2025).

Macrophage-dominated inflammation occurs at 48–96 hours, because an extended neutrophil presence may be (Lu, et al., 2024). Macrophages differentiate from infiltrating monocytes and assume dual roles: M1-type macrophages maintain inflammatory defense by continuing phagocytosis and secrete inflammatory cytokines. While M2-type macrophages facilitate repair by secreting anti-inflammatory cytokines and growth factors like VEGF and epidermal growth factor (EGF) (Lucas, et al., 2010).

The inflammatory stage is not only defensive; it also reshapes the cellular and molecular environment of the wound, preparing it for proliferation and tissue regeneration. Any delay in the inflammatory phase, as is common in diabetic or chronic wounds, can result in delayed healing or fibrosis. (Bainbridge, 2013). Therefore, the regulation of inflammation is critical in determining whether a wound progresses efficiently or stalls in a state of

dysfunction. Additionally, the inflammation phase is controlled by regulatory signals that help terminate the inflammatory response and promote the proliferative phase, such as transforming growth factor-beta (TGF-β), interleukin-10 (IL-10), and lipid mediators (Landén, Li, & Ståhle, 2016).

Proliferative Phase

The third stage is the proliferation stage, during which new tissue is produced to replace damaged or injured tissue. Depending on the degree of the damage and factors such as infection, patient age, vascular supply, overall health, and the condition of the tissue, this phase lasts a few weeks or a few days following the injury. Its defining traits are extensive angiogenesis, extracellular matrix (ECM) remodeling, fibroblast and keratinocyte activation, and migration (Fernández-Guarino, Hernández-Bule, & Bacci, 2023).

One of the key hallmarks of the proliferative phase is re-epithelialization, the process by which keratinocytes, the predominant cells in the epidermis lose cell-cell adhesions, and migrate across the provisional matrix guided by several growth factors, including keratinocyte growth factor (KGF) and epidermal growth factor (EGF). After they migrate across the wound bed, they proliferate to replace lost cells after they cover the wound bed, they begin to differentiate and re-establish stratified epidermal layers (Smith & Rai, 2024).

Fibroblasts move in the direction of the wound and produce important ECM constituents, especially fibronectin, hyaluronic acid, and collagen type III (the initial, less organized collagen that provides a temporary scaffold for granulation tissue, unlike collagen type I, which is stronger and more structured for long-term tissue integrity). These elements strengthen structural integrity for the growing granulation tissue for additional remodeling and cellular attachment (Roman, 2023). Fibroblasts produce collagen type I, a more developed and structured form of collagen that supports long-term tissue strength. (Darby, Laverdet, Bonte, & Desmoulière, 2014).

Another vital feature of the proliferative phase is angiogenesis, which is the formation of new capillaries to ensure oxygen and nutrient supply to the regenerating tissue. This will

sustain the metabolic needs of proliferating and migrating cells, even though newly formed capillaries are fragile, but they're still essential for continued tissue repair. This process is largely driven by endothelial cells that sprout from existing capillaries, vascular endothelial growth factor (VEGF), released by macrophages and fibroblasts in response to hypoxic conditions (Shi, Yao, Shui, Li, & Yan, 2023).

Overall, the proliferation phase is carried out by intercellular signaling, cytokines, and matrix signals, and continues until the wound is fully covered and sufficient new tissue has been formed. While the tissue at this stage is initially disorganized and less functional than the original, the continuing remodeling and maturation improve its structure and strength over time through the action of collagen crosslinking and elastin fiber incorporation (Gardeazabal & Izeta, 2024).

Remodeling Phase

The remodeling phase is the last and longest stage of the wound healing process. Typically begins two to three weeks after injury and lasts up to a year, depending on the patient's health and the size and type of the wound. During this phase, the new tissue undergoes structural refinement to restore the strength and function of the injured tissues. Although the wound may look externally closed, extensive cellular and extracellular activity continues beneath the surface (Fernández-Guarino, Hernández-Bule, & Bacci, 2023).The most important event of this phase is the transition from granulation tissue to scar tissue. Collagen type III, which is more common in the early stages, is gradually replaced by collagen type I, which is thicker, stronger, and more consistent with the skin's structure. (Darby, Laverdet, Bonte, & Desmoulière, 2014).

To achieve appropriate matrix remodeling, matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) control the breakdown of surplus extracellular matrix components (Caley, Martins, & O'Toole, 2015). Myofibroblasts, differentiated from fibroblasts under the influence of TGF-β, play a key role by driving wound contraction and enhancing mechanical strength. Once their function is complete, myofibroblasts undergo apoptosis to prevent excessive scar tissue (Roman, 2023). Additionally, the vascular network developed during

the proliferative phase is refined. leading to a more stable and effective vascular system after apoptosis prunes many of the newly formed blood vessels. The shift from active tissue repair to homeostasis is facilitated by this vasculature normalization (Domenico, 2025).

As remodeling progresses, the collagen fibers in the wound bed better align during this process. Although this procedure is necessary to return the tissue to its natural structure and function, the imbalance between collagen synthesis and degradation may cause scarring. The epidermis returns to its original architecture, completing the re-epithelialization, leading to scar forms, composed mainly of type I collagen and lacking original skin appendages. Hypertrophic scars or keloids can result from degradation and excessive fibrosis, which can affect the functionality of the tissue. (Mathew-Steiner, Roy, & Sen, 2021). Each of the wound healing phase's main events is summarized in Table 2.1:

Table 2.1: Summary of wound healing phases, comparing duration, key events, and cells/factors

Phase	**Duration**	**Key Events**	**Cells/Factors**
Hemostasis	Seconds–Minutes	Vasoconstriction, platelet aggregation, and fibrin clot	Platelets, fibrin, TGF-β, PDGF
Inflammation	Hours–4 days	Pathogen clearance, cytokine release, phagocytosis	Neutrophils, macrophages, IL-1, TNF-α, IL-6
Proliferation	4–21 days	Angiogenesis, fibroblast migration, ECM deposition, re-epithelialization	Fibroblasts, keratinocytes, VEGF, collagen III
Remodeling	3 weeks–1 year	Collagen type III → type I replacement, scar formation, contraction	Myofibroblasts, MMPs, TIMPs, collagen I

Numerous efforts and research studies aimed at restoring tissue integrity, yet the remodeled tissue rarely regains its full strength. Studies show that even under ideal healing conditions, the final scar tissue achieves approximately 70–80% of the original tensile strength of uninjured skin. Unlike species like amphibians and planarians, the ability to completely regenerate damaged tissue without compromising its function is a trait that humans lack, Figure 2.1 highlighting the scarring issue. The dermis in normal skin consists of a mass of randomly oriented, thick collagen fibers, whereas collagen fibers in scar tissue are flattened, oriented in parallel bundles, and thus have a different tissue architecture with less elasticity. The epidermis and keratinocytes are present in both, but scar tissue lacks normal dermal organization. due to human cells having limited plasticity, wounds heal primarily through fibrotic scarring, which makes scarring an inevitable part of wound healing in our species (Khalid, Nawi, Zulkifli, Barkat, & Hadi, 2022).

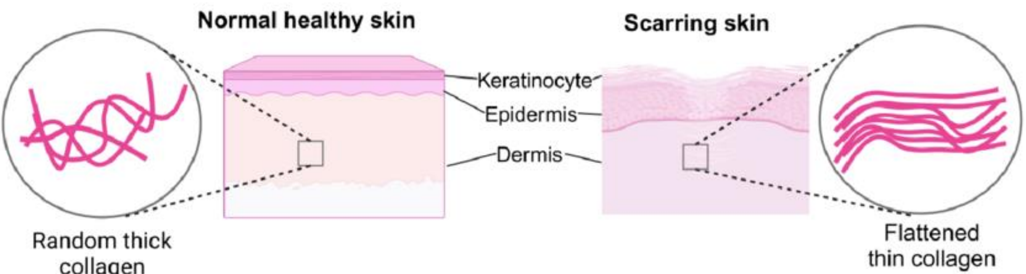

Figure 2.1 Comparison of normal healthy skin and scarred skin (Santoso, Widyantara, Kencana, Setyajati, & Setiawati, 2024)

2.1.2 Classification of Wounds: Acute vs. Chronic

Wounds are classified as acute or chronic based on their underlying pathophysiology and the duration of healing. Acute wounds include conditions such as burns, abrasions, and surgical incisions (Guo & DiPietro, 2010). These typically follow the four-stage wound healing cascade and resolve normally within a predictable timeframe. However, chronic wounds such as diabetic foot ulcers, venous leg ulcers, and pressure sores do not heal normally and often remain in an inflammatory state for a longer time than expected (Wang, et al., 2025). Some distinctions between acute and chronic wounds are outlined in Table 2.2:

Table 2.2: Key differences between acute and chronic wounds.

Feature	**Acute Wounds**	**Chronic Wounds**
Expected Healing Time	Usually heal within 8–12 weeks	Take more than 12 weeks with little or no progress
Primary Causes	Direct trauma, burn, or surgery	Secondary to underlying conditions (e.g., diabetes, vascular disease, immobility)
Inflammatory Response	Controlled and short-lived, resolving as repair begins	Dysregulated and chronic, often suppressing tissue regeneration
Microbial Load	Usually low or absent if the wound is sterile	High risk of infection and biofilm, which slows healing
Tissue Response	Normal granulation tissue formation and re-epithelialization	Impaired angiogenesis, ECM deposition, and epithelial closure

Understanding the differences between acute and chronic wounds is essential as each type requires a different clinical management strategy. While acute wounds often require basic wound care, chronic wounds require a more targeted therapy, which often involves antimicrobial agents, bioactive dressings, or even surgical intervention (Frykberg & Banks, 2015). This also highlights the need to explore an alternative therapeutic approach, such as honey, which has always been known for its role in traditional treatment, especially in the case of skin wounds, whether acute or chronic (Tang, Chen, & Ran, 2024).

2.1.3 Factors Affecting Wound Healing

Many factors affect the wound healing quality and time needed; these factors include Age, nutritional status, diabetes, and other underlying medical conditions that can all significantly impede the healing process. A well-known example of chronic inflammatory

diseases is diabetic foot ulcers, which is one instance of a condition that can impede the proliferative phase and delay recovery by remaining in an inflammatory state for a long time (Wang, et al., 2025). Patients suffer greatly as a result, and physicians face a clinical conundrum. This emphasizes the importance of understanding the molecular mechanisms underlying these phases and creating treatments, particularly for chronic and non-healing wounds.

In addition to systemic influences, local factors at the wound site, such as oxygenation, infection, biofilm formation, and moisture balance, also play a crucial role in determining the healing quality. Oxygenation is vital, as oxygen is required for collagen synthesis, angiogenesis, and resistance to infection; hypoxia is frequently observed in chronic wounds and negatively affects these processes (Tian, et al., 2024). Infection is another critical factor, especially when the wounds develop biofilms; the immune responses in this case will lead to prolonged inflammation and delayed re-epithelialization (Cavallo, et al., 2024).Moisture balance at the wound site impacts healing. A moist wound environment facilitates cell migration, while excessive dryness slows healing or causes tissue maceration. Improper application could potentially lead to excessive moisture or maceration. Additionally, wound size and depth influence healing duration; larger wounds require additional support through dressings or bioactive therapies (Zainuddin, et al., 2025)..

2.2 Honey Through the Ages: Medicinal and Cultural Insights

Honey has been highly treasured for centuries, not only as a sweetening ingredient but also as a medicinal product with unique curative properties. It has a long history in all cultures for wound healing, embalming, and religious practices, thus indicating multiple uses of honey in human lives. Whether it is Egyptian papyrus, Ayurvedic texts, or ancient Greek writing scribbles, honey has been noted for centuries as a natural cure to all sorts of diseases and conditions. An examination of these previous interpretations offers a useful understanding of the century-old tradition of its use as a medicinal agent, and from where its relevance for current biomedical studies arises (Martinotti & Ranzato, 2014).

2.2.1 Medicinal Use of Honey Across Cultures

Honey has been widely reported as a traditional medicine since ancient times, before the discovery of antibiotics and antibacterials. Honey is included in many modern treatments for skin injuries; the historical record supported honey's use as a medicine at least 3500 years ago, and the healing properties of honey on infected wounds by topical application. The ability of honey to achieve this early in wound healing (i.e., by providing a mechanical barrier and antimicrobial cover) was shown with respect to the retardation of the proliferation of pathogenic organisms (Cooper R. , 2007).

Hippocrates, the father of medicine, suggested using honey to treat sores and wounds. In his well-known medical encyclopedia De Materia Medica, Roman physicians like Dioscorides praised honey's therapeutic qualities and suggested using it to treat wounds, sore throats, and eye conditions. Avicenna, a Muslim scholar, advocated skincare, citing its purifying and cleansing qualities in his medical manual, The Canon of Medicine. (Zargaran, Soleymani, Mazhari, & Mirghazanfari, 2024).

In India, Ayurveda (a traditional system of medicine) uses honey to heal digestive problems, wounds, and abscesses. Honey, known as feng mi in traditional Chinese medicine, was applied to the treatment of wounds, cutaneous ulcers, and gastrointestinal diseases. It was also used on surgical wounds and other abscesses and taken by mouth as 'something good for you'. The healing powers of honey were also recognized by the Maya civilization in Mesoamerica (Zumla & Lulat, 1989), as well as throughout Asia and the Mediterranean. The honey produced by stingless bees, known as melipona bee honey, was employed in ceremonial and therapeutic contexts, including wound treatment and eye care.

The global traditions associated with the historical use of honey have emphasized its wide-ranging medical applications, particularly in wound care. This wealth of ethnomedical practice has stimulated contemporary scientific investigations into the antimicrobial, anti-inflammatory, and tissue-regenerating properties of honey, which are associated with several unique physical properties and chemical composition factors (Barazesh P. , et al., 2025).

2.2.2 Honey in Palestine: Cultural and Therapeutic Significance

Due to Palestine's climate and biodiversity, the production of honey has been a significant aspect of its culture for thousands of years. The exceptional therapeutic properties of honey draw the attention of Palestine's traditional and scientific communities. Therefore, Palestinians mainly rely on traditional beekeeping techniques passed down through the generations.

Bees forage the nectar of wildflowers, citrus blossoms, alfalfa (barseem), eucalyptus, *Salvia fruticosa*, and the abundant *Ziziphus spina-christi* tree, which grows in profusion primarily during spring and summer (El-yagoubi, et al., 2025). Each source of nectar imparts special characteristics on the honey, such as flavor, color, consistency, and medicinal properties.

Moreover, various universities and agricultural institutions throughout Palestine are also carrying out some investigations to determine the nutritional contents, antibacterial activities, and antioxidant capacities of different types of honey produced in Palestine (Abu-Farich, et al., 2024). These studies highlight the growing interest of Palestinian researchers in evaluating the potential health benefits of native honey varieties. In other studies, on local natural products, like Thymus capitatus essential oils, have demonstrated notable anti-cancer and antioxidant qualities, indicating that Palestinian scientists are more interested in bioactive local resources (Imtara, Abujaber, Siouri, Tumeh, & Saad, 2025). The medicinal effects of regional honeys, like avocado, alfalfa, and citrus honey, have not been fully investigated, suggesting an opportunity for more targeted research.

A study by Abdulkhaliq and Swaileh (2017) evaluated Palestinian honey against international standards with characteristics such as a pH of 3.44, moisture content of 16.53%, and high levels of reducing sugars (78.86%) and fructose (38.29%) (Abdulkhaliq & Swaileh, 2017). providing evidence of its quality and potential suitability for therapeutic applications.

2.3 Chemical Composition of Honey

Honey is known to exert different bioactive effects in wound healing, which originate from its special physicochemical and biochemical characteristics, including high sugar content, low pH, release of hydrogen peroxide, polyphenolic compounds, and high viscosity (Tashkandi, 2021). The properties are listed in Figure 2.2

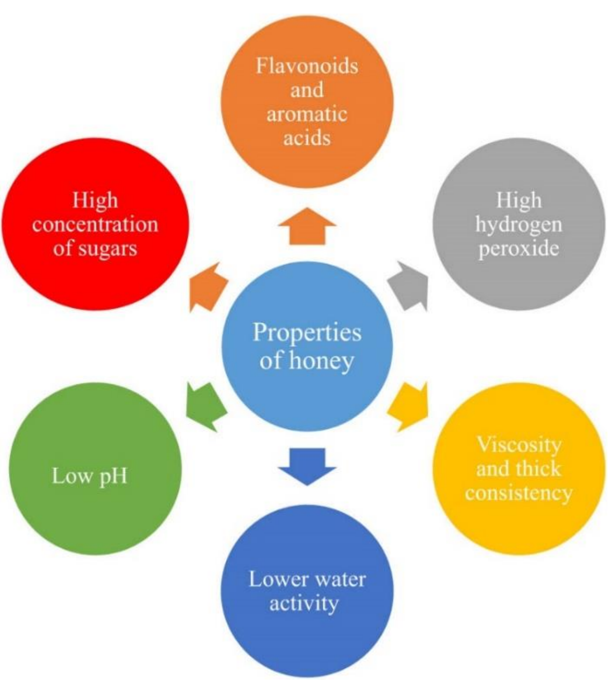

Figure 2.2 : Physicochemical and biochemical essentials of honey for its healing mechanisms (Tashkandi, 2021).

Honey is a complex natural substance, and its composition varies based on floral source, geography, season, and bee species. It is composed of a vast variety of chemical molecules, from the major components (sugars) to minor but extremely bioactive compounds such as phenolic compounds, enzymes, and organic acids (Da Silva, Gauche, Gonzaga, Costa, & Fett, 2016). These constituents not only decide the organoleptic properties of honey but also are responsible for its biological activities via well-known molecular mechanisms. Fig 2.3 highlights the main compounds of the honey

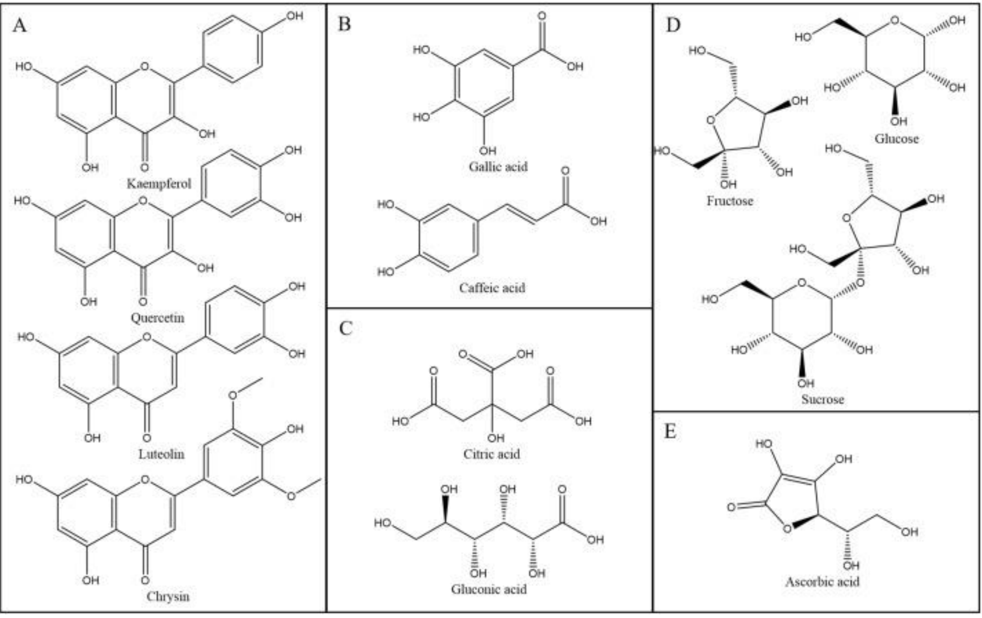

Figure 2.3 : Chemical structures of the major bioactive compounds of honey, including (A) flavonoids, (B) phenolic acids, (C) organic acids, (D) sugars, and (E) vitamins (El-Din, et al., 2025).

Carbohydrates are the main constituents of honey, representing 70–80% of its total weight. It contains fructose (≈ 38%) and glucose (≈31%) as the major monosaccharides, which are responsible for its sweet taste, viscous texture, and hygroscopic property (Da Silva, Gauche, Gonzaga, Costa, & Fett, 2016). Minor sugars are sucrose, maltose, turanose, isomaltose, and melezitose. High sugar content creates an osmotic pressure on microorganisms, which results in microbial growth inhibition.

Glucose is a substrate for the enzyme glucose oxidase. The low-level production of hydrogen peroxide (H2O2) in honey is also more controlled in contrast to the rapid H2O2 release from tissue. The honey's H2O2 controlled release is sufficient to inhibit bacterial growth without being toxic to mammalian extracellular tissue yet bacteriostatic enough to avoid infections, as the enzyme that produces H2O2, glucose oxidase, gives its product a mild antiseptic quality (Naskar, et al., 2024) The process is shown in Fig. 2.4, and this controlled H_2O_2 production is crucial to honey's antimicrobial activity (Almasaudi, 2021).

Figure 2.4: The glucose oxidase reaction that produces H2O2 (Feng, 2023).

Honey does contain proteins (0.1-0.5%), but they appear to be of functional importance. The most common proteins are bee enzymes (invertase, diastase (amylase), glucose oxidase, and catalase). Catalase controls the amount of hydrogen peroxide and balances between antimicrobial and cytotoxic activity. Invertase is an enzyme that splits sucrose into its components, glucose and fructose, which may have an impact on the taste and metabolism of honey.

Honey is believed to contain polyphenols as the single most important bioactive constituent. They consist of flavonoids such as quercetin, kaempferol, luteolin, chrysin, pinobanksin, and pinocembrin, and phenolic acids, also as caffeic acid, ferulic acid, gallic acid, and p-coumaric acid. (Zahra, Abrahamse, & George, 2024). These compounds contribute to honey's bioactivity, which will be discussed in the following section.

Honey holds up to 0.5% of organic acids, the most significant one being gluconic acid, generated by glucose oxidase. This adds to the natural acidity of honey (pH 3.2–4.5) that inhibits the growth of microorganisms and increases mineral solubility as well. Other acids are acetic, citric, lactic, oxalic, and succinic acids, contributing to taste and buffering capacity (Rahman, Hossain, & Barman, 2023).

Despite the fact that they are not present in large quantities, honey contains minerals like potassium, calcium, magnesium, iron, copper, manganese, and zinc. Potassium is often the highest and plays a role in osmotic regulation and cellular metabolism. Vitamins are present in smaller amounts and include ascorbic acid (vitamin C) and members of the B-

complex group (niacin, riboflavin, pantothenic acid). These compounds act synergistically with polyphenols in the antioxidant profile of honey (Sana, et al., 2025).

Amino acids in honey are present in trace amounts, with proline being the most abundant. The content of proline is often employed as a quality and maturity index of honey (Da Silva, Gauche, Gonzaga, Costa, & Fett, 2016). Some types of honey, especially Manuka, contain specific compounds like methylglyoxal (MGO) and leptosperin. MGO is naturally occurring in the nectar of Leptospermum from dihydroxyacetone and accounts for Manuka honey being a non-peroxide antibacterial agent (Majtan, Methylglyoxal—A potential risk factor of manuka honey in healing of diabetic ulcers, 2011).

In summary, honey's chemical nature is complex; although quantitatively sugars predominate its composition, qualitatively enzymes, polyphenols, organic acids, minerals, and other minor forms form a basis for the broad-spectrum healing actions seen in wound care and constitute a mechanistic rationale underpinning many studies that have demonstrated antibacterial, antioxidant, anti-inflammatory, and tissue-regenerative activities.

2.4 Bioactive Properties and Therapeutic Effects of Honey

Honey has been popular since ancient times in the treatment of different diseases, particularly for skin wounds, whether acute or chronic. This is because honey has several properties that differentiate it from other natural options, such as having an antibacterial impact, being a moisture-enhancing agent, and having an anti-inflammatory property (Tricou, Guirguis, Djebbar, Freedman, & Matoori, 2025).

2.4.1 Antibacterial and Antimicrobial Activity

Honey has a lot of antimicrobial properties, working as a physical barrier to the wound from microorganisms. In addition to its properties (80% sugar), it generates a hyperosmolar

environment, which impedes bacterial growth by withdrawing moisture from bacterial cells through an osmotic effect; thus, drying out the wound area. Also, honey's low pH (3.2–4.5) and its inhibitory activity against some common pathogens, including E. coli, P. aeruginosa, and Staphylococcus aureus, and improves tissue oxygenation, which is vital for wound healing (Ogwu & Izah, 2025).

Glucose oxidase catalyzes the reaction of glucose oxidation to gluconic acid and hydrogen peroxide, which is a slow-release antiseptic when diluted (Süntar, et al., 2021) .Shown in Fig. 2.3. Honey also has low water activity, approximately 0.6, and thus impedes microbial life and growth. The vast majority of bacteria need a higher aw to grow, so the low aw environment of honey is simply not conducive to bacterial growth (Maddocks & Jenkins, 2013).

An additional peroxide-independent mechanism that the honey employs is Defensin-1, a honey antimicrobial peptide from bees with broad-spectrum antibacterial activity. Honeybee defensin-1 is synthesized in the honeybee hypopharyngeal gland and secreted into honey and royal jelly. Immunocompetent honeybees produce large amounts of a yellowish fluid (YF), which is likely to contain significant levels of defensin-1 venomous sting secretion, where it plays a very important role in hive health defense (Proaño, et al., 2021). This peptide binds bacterial cell membranes, resulting in cell lysis and blocking the growth of the microbe. Moreover, defensin-1 has been found to have facilitative roles on keratinocyte migration and wound closing, demonstrating its therapeutic contribution to wound healing (Bucekovà, et al., 2017).

Honey has additional synergistic effects that enhance its multiple healing properties. It is relevant to note that the modes of antimicrobial activity of honey are quite dissimilar among its several floral sources. Manuka honey, on the other hand, is known for its extremely high concentration of methylglyoxal (MGO), which accounts for its powerful non-peroxide antibacterial activity (Rabie, Serem, Oberholzer, Gaspar, & Bester, 2016).

On the other hand, Palestinian honeys, Citrus, Alfalfa, and Avocado, are reported by polyphenol-mediated and hydrogen peroxide–mediated activities singly or combined with

organic acids, as well as osmotic effect. This mechanistic difference highlights the importance of characterizing these honeys; the difference between honeys may confer different benefits for wound healing purposes. (Molan, 2001).

2.4.2 Antioxidant Properties

Honey contains a lot of polyphenolic compounds, particularly flavonoids and phenolic acids. These compounds are crucial for honey's biological effects, particularly in wound healing. They provide antioxidant activity, which helps get rid of free radicals that could damage cells. Additionally, they possess anti-inflammatory properties that reduce the expression of pro-inflammatory cytokines. Consequently, the environment is more conducive to tissue healing and repair (Wilczyńska & Żak, 2024). Along with glucose oxidase, honey also contains enzymes like catalase, invertase, and diastase that help break down carbohydrates, improve nutrient absorption, and maintain wound site homeostasis (Farooq & Ngaini, 2023).

From a mechanism perspective, these molecules work in several ways. First, they donate electrons and scavenge radicals to defend against reactive oxygen species (ROS), (Santos, Maia, Barros, & Gouvinhas, 2023). Highlighted in Fig. 2.5

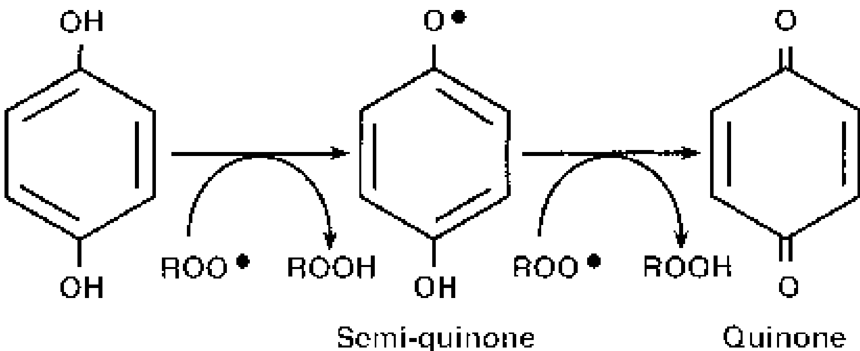

Figure 2.5: hydrogen donation by a phenolic antioxidant to lipid. The resulting unpaired electron becomes delocalized within the ring structure so that the phenolic radical is relatively unreactive before ultimately forming a quinone (Duthie, 1999).

Second, they chelate metal ions, inhibiting Fenton reaction–induced ROS production. Hydrogen peroxide can be decomposed by traces of redox-active metals such as copper and iron to form a hydroxyl radical by undergoing the Fenton reaction. Hydroxyl radicals are among the most reactive species in biological systems and are responsible for damage to biomolecules. At physiological levels, ROS act as signaling molecules that maintain many important physiological functions (Kejík, et al., 2021). This process is highlighted in Fig.2.6

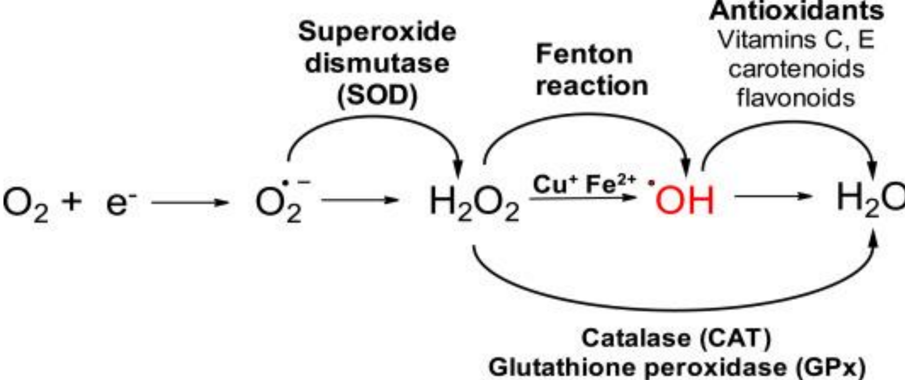

Figure 2.6: Flavonoids chelation of transition metal ions, stopping the production of Hydroxyl radicals (Jomova, et al., 2025).

Additionally, these molecules modulate signaling pathways by inhibiting NF-κB and activating Nrf2, resulting in suppression of inflammatory and oxidative responses (Alvarez-Suarez, et al., 2016).

The structural features of flavonoids, such as Hydroxyl groups and conjugated double bonds, are structural determinants of flavonoids, which facilitate the honey's ability to donate electrons, forming stable free radicals, reflecting the honey’s redox-modulating capacity.

These bioactive compounds are also reflected in the color of honey, which ranges from pale amber to dark brown, and have invariably been correlated with variation in bioactive content, with dark honeys having a higher phenolic and flavonoid content and higher antioxidant activity than light honeys. A review refers to a strong positive correlation between

color intensity and antioxidant activity, and also between color and total phenolic content (Becerril-Sánchez, Quintero-Salazar, Dublán-García, & Escalona-Buendía, 2021).

2.4.3 Anti-Inflammatory and Immunomodulatory Effects

Honey moderates the inflammation that occurs after the injury by preventing an excessive or uncontrolled inflammatory response, which may lead to tissue damage and result in a chronic wound. This is achieved through downregulation of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, while at the same time promoting the release of anti-inflammatory mediators that lead to the wound transitioning from the inflammatory to proliferative phase of the wound healing process. This balanced modulation prevents immune hyperactivation and fosters orderly tissue healing (Iosageanu A. , Stefan, Craciunescu, & Cimpean, 2024).

It has been recently shown that honey can regulate macrophage phenotype and function to an extent that is conducive to tissue repair and inflammation resolution. For instance, Manuka honey was found to prevent LPS-mediated injury in macrophages through improving mitochondrial function, decreasing apoptosis, and inhibiting inflammatory signaling (p38, ERK1/2) and up-regulation of AMPK, SIRT1, and PGC-1α (Afrin, et al., 2018).

One of the mechanisms through which these actions are generated is through honey's antagonism against oxidative stress. Macrophages and neutrophils, during inflammation, produce reactive oxygen species (ROS) that, in excess, sustain cytokine release by NF-κB and MAPK pathways, forming an inflammatory vicious cycle (Wani, et al., 2020). Flavonoids and phenolic compounds present in honey neutralize ROS and reduce oxidative signaling, and hence inhibit inflammatory cascades and protect host cells from oxidative injury. This bidirectional interaction not only inhibits tissue damage but also promotes an immune environment that is conducive to resolution and healing (Tricou, Guirguis, Djebbar, Freedman, & Matoori, 2025).

2.4.4 Effects on Cell Proliferation and Re-epithelialization

When the skin is injured, the protective outer layer of skin (the epidermis) is disrupted. To repair this damage, the keratinocytes, which are the primary cells in the epidermis, must undergo the phases of wound healing. Proliferation refers to the ability of basal keratinocytes to divide and multiply, increasing the number of keratinocytes available to cover the wound area (Kamysz & Kleczkowska, 2025). They are influenced by various growth factors, cytokines, and signaling pathways, such as transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α), and epidermal growth factor (EGF). These signals stimulate keratinocytes to activate, change gene expression, and produce enzymes such as matrix metalloproteinases (MMPs).

Several studies suggest that honey can modulate the expression of important cytokines in keratinocytes, such as interleukin-1β (IL-1β), tumor necrosis factor-alpha (TNF-α), and transforming growth factor-beta (TGF-β) (Tashkandi, 2021). These molecules are responsible for regulating the keratinocyte behavior during the wound healing process.

2.4.5 Promotion of Angiogenesis and ECM Formation

Honey characteristics include encouraging the synthesis of collagen and supporting the formation of the extracellular matrix, enhancing the strength of the new tissue formed in the wound healing process (Ibrahim, Thangavelu, & Khalifa, 2022). Additionally, it promotes angiogenesis, which ensures an adequate flow of oxygen and nutrients to support tissue repair and cell proliferation, thereby facilitating the development of new blood vessels (Majtan, Methylglyoxal—A potential risk factor of manuka honey in healing of diabetic ulcers, 2011). By creating an oxidative environment and restoring equilibrium, honey aids in the healing of wounds and helps them return to the proliferative phase (Al-Waili & Al-Waili, 2011). These properties are summarized in Table 2.3

Table 2.3: Bioactive Properties of Honey and Their Contribution to Wound Healing

Property	**Key Mechanism**	**Outcome in Wound Healing**
Antibacterial & Antimicrobial	High sugar content → osmotic effect; low pH; hydrogen peroxide release; methylglyoxal (non-peroxide activity)	Inhibits pathogen growth, reduces infection, and accelerates wound sterilization
Antioxidant	Polyphenols (flavonoids, phenolic acids); enzymes (catalase, glucose oxidase) → neutralize free radicals	Reduces oxidative damage, protects tissue, and creates a favorable healing environment
Anti-inflammatory & Immunomodulatory	Downregulates TNF-α, IL-1β, IL-6; induces anti-inflammatory mediators; balances neutrophil activity	Controls excessive inflammation, prevents chronic wounds, and promotes a balanced immune response
Cell Proliferation & Re-epithelialization	Modulates keratinocyte cytokines (IL-1β, TNF-α, TGF-β); increases MMP-2, ERK1/2 activity	Enhances keratinocyte and fibroblast migration, supports re-epithelialization, and wound closure

Together, these bioactive processes contribute to the transition of honey from traditional medicine to evidence-based wound care. It is the anti-microbial, anti-oxidative stress, inflammatory modulating, and cellular repair-stimulating activities of honey that are physiologically expressed as healing acceleration, infection prevention, and patient comfort (Gurtner, Werner, Barrandon, & Longaker, 2008). In this way, the honey dressings are becoming more and more the first line of defense in everyday clinic therapy for burns, ulcers, and non-healing wounds, thus confirming the role of their multitargeted therapeutic activities (Kondej, et al., 2024).

2.5 Experimental Evidence on Honey's Effects in Wound Healing

For the past 20 years, there has been a long focus on in vivo and in vitro to evaluate the therapeutic value of honey. Such investigations lend powerful support for the use of honey as a wound-healing agent with its inflammation modulation, re-epithelization potentiation, fibroblast and keratinocyte stimulation, and microbial colonization inhibition. The full spectrum of in vitro evidence observed from testing performed under controlled experimental conditions is discussed in the subsequent subsections, i.e., keratinocyte-derived models and assays involving immune-response (THP-1) (Miraldi, et al., 2024).

2.5.1 In Vitro and In Vivo Evidence of Honey in Wound Healing

A prospective clinical trial which compared the application of honey as a dressing in open wounds or those infected with other wound dressings in patients found that at 1 week, there was reduced bacterial contamination, as demonstrated by positive wound cultures decreasing from 55% to 23%. In the first 2 weeks, the median rate of wound healing was 3.8 cm^2 per week; this constitutes a very good level of tissue regrowth. By week three, 86% of the patients deemed dressing pain-free as an indicator that honey facilitates patient comfort (Mphande, Killowe, Phalira, Jones, & Harrison, 2007) .

Another clinical trial compared the use of honey dressings and silver sulfadiazine in 104 patients with superficial burns. In the honey group, 91% of wounds were sterile after seven days, compared to 7% in the silver sulfadiazine group. Granulation tissue developed faster in the honey group, and 87% of wounds were healed after 15 days, compared to 10% of controls. Honey dressing was also less painful, caused fewer hypertrophic scars, and a reduced chance of post-burn complications, demonstrating honey's value as a cheap and available burn dressing (Subrahmanyam, 1991) .

A randomized controlled trial of Manuka honey vs.IntraSite Gel for sloughy venous leg ulcers found more slough reduction (67% vs.53%) and a significantly greater reduction in wound size (34% vs.13%) at four weeks in the honey group. At 12 weeks, 44% of the wounds treated with honey healed versus 33% with the gel. Infections were fewer in the honey group. These findings support the application of Manuka honey in desloughing, healing promotion, and infection minimization in venous ulcers (Gethin & Cowman, 2009) .

2.5.2 Honey Effects on Keratinocyte Proliferation and Migration

Keratinocytes have a primary function in re-epithelialization of injured skin, and their responses to honey are increasingly being investigated in vitro. Several reports utilizing HaCaT keratinocyte cell lines indicate that honey enhances cell viability, scratch wound migration, and modulates cytokine and growth factor secretion (Liu, Zeng, Li, Ke, & Xu, 2022).

A comparison between the effects of Manuka honey (MHn) and Indonesian Trigona honey (THn) on fibroblasts and keratinocytes of patients with chronic otitis media was done by Priyono et al .Cells were incubated with various honey concentrations (0.04%, 0.1%, 0.25%) and tested for proliferation and keratinocyte growth factor (KGF) and basic fibroblast growth factor (bFGF) secretion. Honey had no impact on the quantity of fibroblasts but reduced their doubling time, which is indicative of faster division.

Keratinocyte populations increased markedly in all MHn groups, and the lowest THn concentration released was cell number-dependent, particularly on days 6 and 8, while bFGF decreased with proliferation. The duration of exposure to honey was positively related to cell proliferation, and it signifies that both honeys support keratinocyte proliferation with wound healing activity (Priyono, et al., In vitro activity of Manuka and Trigona honey on fibroblast and keratinocyte cultures, 2024) .

Another experiment evaluated jamun honey polyphenolic extracts (HEP) on HaCaT cells under hypoxia.HEP alleviated the cytotoxicity and enhanced cell viability at an optimum concentration (0.063 mg/mL). Molecular assays showed the revived expression of adhesion and proliferation markers (E-cadherin, β-catenin, Ki67), and spectral results showed preserved cellular integrity. These results suggest that jamun honey polyphenols are protecting against hypoxia-mediated keratinocyte injury, validating their therapeutic roles in wound healing under stress conditions (Chaudhary A. , Bag, Banerjee, & Chatterjee, 2017) .

A comparison of acacia, buckwheat, and manuka honeys on HaCaT cell re-epithelialization demonstrated that all enhanced wound closure and migration at 0.1% concentration. Molecular signaling, however, varied: all activated cyclin-dependent kinase 2 and focal adhesion kinase, while activation of vasodilator-stimulated phosphoprotein, integrin-β3, cdc25C, and MAPK pathways differed by honey type. Interestingly, epithelial-mesenchymal transition (EMT) induction also varied, with acacia and buckwheat influencing more EMT-related genes than manuka. This implies that the botanical origin of honey influences specific molecular mechanisms in keratinocyte-mediated repair (Ranzato, Martinotti, & Burlando, 2012) .

Despite these findings, comparisons between different monofloral honeys, especially from less-represented regions like Palestine, are limited. Most studies deal with well-known medicinal honeys like Manuka or Tualang. Thus, Citrus, Avocado, and Alfalfa honey testing on keratinocytes fills a broad research niche.

2.5.3 Honey and Inflammation: THP-1 Macrophage Models

Besides promoting regeneration, honey also controls inflammation, which is a vital phase of wound healing (Iosageanu A. , Stefan, Craciunescu, & Cimpean, 2024). The human monocytic THP-1 cells, differentiated into macrophage-like cells, are a well-standardized in vitro model for studying the impact of honey on inflammatory mediators such as nitric oxide and cytokines (Alqarni, et al., 2019). There has been in vitro work highlighting the multitarget therapeutic potential of certain local honeys, e.g., Hyphaene thebaica, to regulate the

macrophage activity and inflammation (Abu-Farich, et al., In vitro evaluation of Hyphaene thebaica honey as a multitarget therapeutic product, 2025). Similar research with other bioactive natural compounds, such as Thymus capitatus essential oils, has also offered evidence for antioxidant and immunomodulatory activity, which shows a broader interest in Palestinian local natural products in wound treatment (Imtara, Abujaber, Siouri, Tumeh, & Saad, 2025).

Collectively, these investigations illustrate THP-1 macrophage models as useful platforms for studying honey's immunomodulatory activity, offering molecular-level insight into honey's impact on macrophage function data essential to understanding its therapeutic value in wound healing.

A study used the MonoMac-6 (MM6) monocytic cell line to examine manuka, pasture, and jelly bush honeys at a concentration of 1% for their effects on inflammatory cytokine release (TNF-α, IL-1β, IL-6). All honeys significantly increased cytokine production compared to untreated and artificial honey controls, with jelly bush honey inducing the highest levels. (Tonks, et al., 2003) .

This suggests that honey can facilitate wound healing by partly stimulating monocytic cells to release pro-inflammatory cytokines required for tissue repair and infection resolution. But little research has been done on Palestinian honeys in this regard. The present study uses the THP-1 cell line to examine Citrus, Avocado, and Alfalfa honeys' anti-inflammatory effects through nitric oxide measurement, complementing keratinocyte assay results.

2.6 Molecular Targets and Pathways Modulated by Honey in Wound Healing

2.6.1 Molecular Targets of Honey in Wound Healing

Honey is rich in bioactive molecules, including polyphenols and flavonoids, which are believed to target specific molecular pathways involved in the healing process. The mode of

action is mainly through the induction of antioxidant and anti-inflammatory cascades controlling cellular processes involved in tissue regeneration.

1. Antioxidant Enzymes:

- Heme Oxygenase-1 (HO-1): Polyphenolics present in honey were found to activate the Nrf2/ARE signaling pathway, which resulted in the expression of HO-1. It is an enzyme involved in cytoprotection against oxidative stress by the degradation of heme to biliverdin, iron, and carbon monoxide, antioxidant and anti-inflammatory end products. We feel that our findings are consistent with this activity of the protein (Süntar, et al., 2021).

- NAD(P)H Quinone Dehydrogenase 1- (NQO1): Honey treatment promotes NQO1 expression through an activating influence of Nrf2. NQO1 is one of the detoxification enzymes that catabolize quinones and scavenge ROS in cells, leading to suppression of cellular oxidative stress (Jeong, et al., 2019).

2. Inflammatory Mediators:

- Inducible Nitric Oxide Synthase (iNOS): Honey has been found to impact the expression of iNOS. iNOS generates NO, the overexpression of which is inflammatory and tissue-destructive. Honey suppresses iNOS and also not only NO synthesis but also the inflammatory reaction coupled with wound healing (Silva, et al., 2021).

- Cyclooxygenase-2 (COX-2): COX-2 is a pro-inflammatory mediator that is engaged in the synthesis of pro-inflammatory prostaglandins. Anti-inflammation caused by honey is also due to downregulation of the expression of COX-2 and consequent inhibition of the synthesis of pro-inflammatory prostaglandins, resulting in anti-inflammation at the injury site (Ahmed, Al Nohair, & Abdulmonem, 2022).

3. Apoptotic Regulators:

- B-cell lymphoma 2 (Bcl-2): The second cell survival-regulating protein. Honey controls the expression of an anti-apoptotic gene (Bcl-2), thereby regulating cell survival while at the same time having an activity on excessive cell death, an important process in the reconstitution of tissue in wound healing (Bangash, et al., 2024).

- Caspases: A family of proteases with a role in cellular apoptosis. Regulation of caspase activity in honey may regulate apoptosis to prevent the clearing of damaged cells but not to inhibit wound closure as a whole (Rahmani & Babiker, 2025).

4. Extracellular Matrix Remodeling:

- Matrix Metalloproteinases (MMPs): MMPs comprise a group of enzymes that degrade the extracellular matrix. Honey could affect matrix metalloproteinases (MMPs) and the roles of the extracellular matrix in wound healing and tissue repair (Majtan, et al., 2013).

2.6.2 The Molecular Pathways Regulated by Honey

The wound healing process affected by honey is not only mediated by its physical and chemical properties but also modulated by a number of molecular signaling pathways in tissue repair, resolution of inflammation, and cell growth. Its activities span various stages of healing, such as inflammation, proliferation, and remodeling, through modulating intracellular events in immune cells, keratinocytes, and fibroblasts.

1. NF-κB Pathway: Honey suppresses the NF-κB, the primary pathway, a key mediator in inflammation. This blockade results in the suppressed expression of pro-

inflammatory cytokines, including TNF-α, IL-1β, and IL-6, promoting the shift from inflammation to wound healing's proliferative phase (Alvarez-Suarez, et al., 2016).

2. MAPK/ERK Pathway: Honey mediates the activation of the MAPK/ERK pathway, which serves in the process of cell proliferation and differentiation. This activation is associated with re-epithelialization and granulation tissue formation in wound healing (Wani, et al., 2020).

3. PI3K/Akt Pathway: Honey activates the PI3K/Akt pathway, which stimulates cell survival, migration, and angiogenesis. Such activation leads to tissue regeneration and wound healing (Ranzato, Martinotti, & Burlando, 2013).

4. Nrf2/HO-1 Signaling Pathway: Honey triggers the Nrf2/ARE pathway to increase antioxidant enzymes such as HO-1. This activation decreases oxidative stress and inflammation in order to enhance tissue repair (Süntar, et al., 2021).

2.7 Comparative Analysis of Honey Types and Their Biological Activities

The biological activity of honey varies significantly based on its botanical origin, where floral sources contain various chemical profiles and therapeutic activity (Jerković, et al., 2025). The Palestinian honeys picked for our study are Citrus, Alfalfa, and Avocado honeys. The reason for picking these types of honey will be highlighted in this section.

Additionally, a comparison of these honeys' literature is discussed, as it allows both the evaluation of locally produced varieties with potential clinical use and a comparison against an internationally known standard. The current section, therefore, presents an overview of the origin, phytochemical profile, and medicinal virtues of each honey variety, representing the scientific and practical justification for their selection in the current research.

2.7.1 Citrus Honey

2.7.1.1 Origin and Composition

The main source of citrus honey is the nectar from citrus flowers like orange, lemon, and grapefruit trees. Citrus plants are nectar-bearing plants, which makes them preferred by bees due to this property. Citrus honey is found in big counties in Mediterranean and subtropical regions, where citrus trees are (Karabagias & Karabournioti, 2018) .

The citrus honey contains a high percentage of simple sugars such as fructose and glucose, which make up about 70-80% of its composition. Apart from that, various constituents, including minerals, phenolic and volatile components, amino acids, sugars, enzymes, vitamins, methylglyoxal, and organic acids, are also found in citrus honey. Citrus honey has a different phenolic acid composition than other honeys, but that's what gives it its unique antioxidant and medicinal qualities. In citrus honey, important phenolic acids include p-coumaric acid, syringic acid, and caffeic acid (Lazaridis, Kitsios, Koutoulis, Malisova, & Karabagias, 2024).

The predominant phenolic compounds in citrus honey are hesperetin and hesperidin, rutin, abscisic acid, 3,4-dimethoxycinnamic acid, as well as comparatively large amounts of p-coumaric acid, caffeic acid, quercetin, and kaempferol. Among these, hesperetin and its glycoside hesperidin are considered the main bioactive markers that not only reflect citrus honey's botanical origin but also contribute in no small measure to its free radical scavenging and anti-inflammatory characteristics. Citrus honey is generally acidic in nature, with a pH between 3.5 and 4.5, which is responsible for its antimicrobial action (Seraglio, et al., 2021).

Whilst citrus honey is generally lighter and has a lower TPC than darker honeys, it is unique in that its high flavanone content endows it with potent biological effects and lends support to its medicinal potential (Fratianni F. , Amato, d’Acierno, De Feo, & Nazzaro, 2023).

2.7.1.2 Ethnomedicinal and Clinical Relevance

Honey has been applied for centuries as a natural treatment for wounds, burns, and other types of skin injury. Around the world, practices have been developed adding a topical application of honey to aid in the healing process and prevent secondary bacterial infection, as well as speeding up the rate of repair of damaged tissues. In a more modern clinical and experimental environment, such findings about honey's effects have, of late, been increasingly corroborated in vivo with in vitro studies. These confirm that the beneficial uses of honey for sores appear due to its ability to help close up wounds, promote angiogenesis, and kickstart collagen synthesis.

For example, an experimental study evaluated the orange honey by testing its healing effects. The spring orange honey was tested on rats. Thirty-two rats were anesthetized and subjected to full-thickness excisional wounds measuring 1 cm. The wounds were treated with honey twice daily for 14 days. Histological analysis revealed enhanced angiogenesis, collagen synthesis, and epithelial formation in the honey-treated group. By day 14, wounds showed thicker, more organized collagen fibers and complete closure. Compared to phenytoin 1%, citrus honey promoted faster tissue regeneration. These results support its potential as a natural wound healing agent (Pourmahdi, Moshtagh, Rezvan, & Gholami-Ahangaran, 2022)

2.7.1.3 Insights from In Vitro Cellular Studies

Despite the lack of in vitro research into cellular processes influenced by Citrus honey during wound healing, in vitro findings have, in recent years, indicated potential bioactivity in cellular models. Citrus honey has been reported to stimulate proliferation and migration in human keratinocytes and fibroblasts in vitro.

Study by (Governa, Carullo, Biagi, Rago, & Aiello, 2019) employed a scratch wound-healing assay to determine the regenerative ability of various Calabrian honeys and their extracts on HaCaT keratinocytes. However, follow-up proliferation assays revealed that these samples did not induce an increase in keratinocyte proliferation at 6 hours or 24 hours after treatment compared to controls. Interestingly, during corticosteroid-induced proliferation suppression, these honey extracts retained a wound-healing effect without causing

cytotoxicity, as evidenced by remaining LDH levels. This suggests that pro-healing activity could be mediated by migration and perhaps anti-inflammatory pathways, and not by direct stimulation of cell growth**.**

2.7.1.4 Antimicrobial Activity

Citrus honey has excellent antimicrobial activity against most Gram-positive and Gram-negative bacteria that are most commonly involved in wound infection. Recent in vitro studies have demonstrated that Citrus honeys have good antimicrobial activity, particularly against biofilm-producing pathogenic bacteria. The honeys were reported to inhibit the growth and metabolic processes of biofilms formed by Listeria monocytogenes, Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, and Escherichia coli. Their high sugar content predisposes to osmotic stress in microbes, acidic pH, generation of hydrogen peroxide by glucose oxidase, and bioactive phytochemicals such as flavonoids with bacteriostatic effect (Fratianni F. , Amato, d'Acierno, De Feo, & Nazzaro, 2023).

2.7.1.5 Antioxidant Potential and Phytochemical Content

Citrus honey is generally characterized by its lighter color and lower antioxidant capacity compared to darker honeys. A study by Tananaki et al. (2024) reported that citrus honeys exhibited both the lowest mean total phenolic content (65.0 ± 15.0 mg GAE/100 g) and the lowest mean total antioxidant activity (6.4 ± 3.5 mg AAE/100 g) among nine monofloral honey types analyzed (Tananaki, Rodopoulou, Dimou, Kanelis, & Liolios, 2024). The antioxidant activity of this honey is correlated with its total polyphenol, flavonoid, and vitamin C content (Lazaridis, Kitsios, Koutoulis, Malisova, & Karabagias, 2024)..

Among the types tested, lime honey contained the highest antioxidant activity, and also the highest polyphenol and vitamin C contents. Lemon and tangerine honeys, however, were dominated by flavonoid composition. In addition to radical-scavenging activity, these honeys showed moderate inhibition of oxidative stress- and neurodegenerative process-involving enzymes such as acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tyrosinase

with inhibitions of up to 12.04%, 19.11%, and 94.1%, respectively. Such findings support the use of citrus honeys as natural antioxidant agents with therapeutic promise (Fratianni F. , Amato, d'Acierno, De Feo, & Nazzaro, 2023).

2.7.1.6 Summary and Knowledge Gaps

Citrus honey has a decent number of biological activities that are very beneficial in healing wounds. It is antimicrobial as a result of its high sugar, low pH, hydrogen peroxide production, and bioavailability of phytochemicals such as flavonoids. A lot of in vivo experiments confirm that citrus honey is capable of accelerating tissue regeneration rate, enhancing collagen synthesis, and promoting re-epithelialization. In vitro data also suggest that its wound-healing action may be mediated by enhanced cell migration and not directly by proliferation. Its antioxidant which is a lower than other honeys ,content, also helps in mitigating oxidative stress at the wound site. Such composite properties make citrus honey an excellent choice for integrative wound healing strategies.

Nevertheless, in the face of all previous positive effects, there are currently some gaps in the literature that address the potential applications of citrus honey on wound healing:

- Limited in vitro evaluations of the direct effect of citrus honey on human skin cells proliferation and wound healing mechanisms in depth.

- Lack of studies comparing citrus honey with other monofloral honeys to show its impact on skin cells' viability, migration, and antioxidant properties.

- The requirement for in vitro assays to assess the correlation between antioxidant profile and cellular responses on wound models.

- No clinical trials are confirming its therapeutic utility in healing human wounds.

2.7.2 Alfalfa Honey

2.7.2.1 Origin and Composition

Alfalfa honey is primarily derived from the nectar of Medicago sativa (alfalfa) flowers, a leguminous plant widely cultivated for forage. These flowers are very rich in nectar, which attracts bees during peak bloom periods in mid-summer, making the honey affordable. Alfalfa honey is widely found in North America, particularly Canada and the United States, where alfalfa fields are extensively cultivated.

Like any other monofloral honey, alfalfa honey consists mainly of simple sugars such as glucose and fructose, which account for 70–80% of the honey. It contains a chain of minor but biologically active components like organic acids, minerals, amino acids, vitamins (especially vitamin C), and volatile compounds. Yet Alfalfa honey has a very rich polyphenolic profile, which underpins its notable antioxidant and therapeutic properties. It contains a variety of phenolic acids such as caffeic acid, p-coumaric acid, caftaric acid, and chlorogenic acid, alongside flavonoids including kaempferol and quercetin. These compounds work synergistically to scavenge reactive oxygen species (ROS) (Fratianni F. , et al., 2024).

Alfalfa honey is known for its rich content of flavonoids. This gives the honey antioxidant and anti-inflammatory properties. Key flavonoids identified in alfalfa honey include kaempferol, quercetin, and isorhamnetin. These compounds have a great influence on the reduction of ROS (reactive oxygen species), and they help to prevent the damage caused by resultant oxidative stress, promoting a healthy constitution. (Bhagwat, Haytowitz, & Holden, 2011). The flavonoid profile of alfalfa honey can vary among individual samples due to differences in floral source and processing methods, yet it consistently enhances the bioactive molecules index of each honey. Additionally, it has been demonstrated through recent research that alfalfa honey presents a high total polyphenol content (TPC), as high as 408 µg/g, which is the highest among five Italian leguminous honeys studied (Fratianni, et al., 2023).

2.7.2.2 Ethnomedicinal and Clinical Relevance

Even though alfalfa honey has not been published well in clinical wound care as citrus or manuka honey, participants of Alfalfa (Medicago sativa) have been investigated on traditional and experimental bases for their wound-healing effects. Alfalfa extracts have also been reported to enhance re-epithelialization and tissue repair because, in animal studies, it has been observed that they stimulate fibroblast proliferation, collagen production, and granulation tissue formation (Suwanto & Hussaana, 2023).

Although the clinical direct evidence for alfalfa honey is scarce, these results at least partly indicate that alfalfa bioactive compounds may, at least partly, explain why its honey has been used anecdotally as a skin inflammation calming agent and burn-healing adjuvant or to support minor wound repair.

2.7.2.3 Insights from In Vitro Cellular Studies

Direct research about alfalfa and keratinocytes was not found, but other studies, including alfalfa-derived biomaterials, show enhanced cellular proliferation. Alfalfa extracts had significant potential in the modulation of cellular processes that are pivotal for effective wound healing. In an experimental study done in a controlled experiment on Wistar rats, by Suwanto and Hussaana (2023), the effect of different concentrations of alfalfa extract (10%, 20%, and 30%) on wound healing parameters was investigated.

The study measured macrophage and fibroblast numbers on day 3 post-treatment and collagen synthesis on day 7. The outcomes showed that treatment with 20% alfalfa extract promoted fibroblast proliferation more significantly compared to lower concentrations (10%) and the positive control (gentamicin) groups. Fibroblast proliferation is significant since such cells release the components of the extracellular matrix, which give structure to the new tissue scaffold. Furthermore, deposition of collagen fibers, a key to wound strength and remodeling, was elevated by 20% and 30% alfalfa over controls.

Meanwhile, macrophage numbers were reduced, suggesting alfalfa extract supports modulation of the inflammatory process to avoid chronic inflammation and smooth transition into healing. These findings indicate that alfalfa extract accelerates the healing of wounds by promoting cell growth and matrix accumulation while inhibiting inflammation, indicating its use as a natural medicament in wound care (Suwanto & Hussaana, 2023).

2.7.2.4 Antimicrobial Activity

Alfalfa honey exhibits mild antibacterial activity because of its sugar content, low water, and phenolic composition. Not so much information about the antimicrobial properties of honey is found in the literature. The chief antimicrobial mechanism comes from the release of H2O2 (hydrogen peroxide), as spectrophotometric tests support, and factors other than peroxides, such as its low water content, pH, and phenolic compounds, also work with this honey to keep away bacteria. (Bravo & Santhoff, 2024). These characteristics suggest that tallow honey could prove promising for treating infections and assisting in the healing of wounds resistant to traditional antibiotics.

Comparative tests on five leguminous honeys indicated strong anti-biofilm activity of alfalfa honey against Acinetobacter baumannii, a commonly associated pathogen with chronic wound infections and hospital-acquired diseases. The inhibitory rates were substantial, with biofilm inhibition up to 81.71% in some cases. Such findings support the possible application of alfalfa honey in the prevention of bacterial colonization and biofilm development, both of which are central to the management of infected wounds. Such biofilm-inhibiting activity was comparable to other leguminous honeys, such as astragalus honey, suggesting the therapeutic potential of alfalfa honey in the treatment of antibiotic-resistant infections (Fratianni, et al., 2023).

2.7.2.5 Antioxidant Potential and Phytochemical Content

Alfalfa honey possesses interesting antioxidant activity that is mainly attributable to its phytochemical content, such as phenolic acids, flavonoids, and other minor bioactive

compounds. These molecules work as free radical scavengers, chelators of transition metals, and modulators of oxidative stress pathways.

Phenolic acids, such as caffeic, ferulic, and p-coumaric acids, are of special interest because they can quench reactive oxygen species (ROS), block lipid peroxidation, and stabilize membranes. Another class of compounds, flavonoids, including quercetin, kaempferol, and luteolin, boost the antioxidant defense system either by handing over hydrogen atoms or electrons to reactive species, as well as by stimulating endogenous antioxidant enzymes.

The antioxidant capacity of alfalfa honey could be due to its phytochemical content, which is parallel with the richness in phytochemicals of Medicago sativa. Information from the USDA Database for Flavonoid Content points out that alfalfa is rich in a broad range of polyphenolic compounds, which directly result in its antioxidant (Bhagwat, Haytowitz, & Holden, 2011). These bioactive compounds not only scavenge ROS but also protect proteins, lipids, and nucleic acids from oxidative damage, a scenario of paramount importance during tissue repair and wound healing.

For example, a survey of 53 commercially available Iranian honeys in mass demonstrated satisfactory contents of quality markers such as moisture, reducing sugars, and proline, and could be quantified for antioxidant activity using the DPPH radical scavenging and FRAP assays (Zarei, Fazlara, & Hamedani, 2019).58.98 mg/mL in the DPPH assay, better than the results reported previously with values >80 mg/mL. Although its antioxidant activity is less powerful than that of other leguminous honeys such as sainfoin (IC50 = 6.08 mg/mL) and carob honey (IC50 = 19.26 mg/mL), it still shows certain free radical scavenging capacity.

The variation in reported antioxidant activity likely is a reflection of differences in botanical source, geographical location, or climate. Significantly, although alfalfa honey is not among the honeys of highest antioxidant activity, its moderate activity is of biological importance. By its capacity to reduce oxidative stress, it can be implicated in anti-

inflammatory reactions and cell protection during wounding conditions, and therefore, it is a valuable adjunct to natural wound management approaches.

2.7.2.6 Summary and Knowledge Gaps

Alfalfa honey exhibits favorable biological characteristics towards its therapeutic activity in wound healing and infection avoidance. Both in vivo and in vitro studies supported the evidence of the wound healing activity of alfalfa honey. Since the alfalfa honey has been shown to cause fibroblast proliferation, increase collagen fiber deposition, and restrict the incidence of macrophages in wound models, making tissue reconstruction, along with inflammatory regulation, possible.

Additionally, antimicrobial experiments prove that alfalfa honey has notable biofilm inhibition against pathogens like Acinetobacter baumannii, with a potential application in the treatment of chronic wound infection and prevention of bacterial colonization. Though more clinical research is required, these findings bear witness to the utility of alfalfa honey as a natural treatment for wounds, especially chronic wounds and infection prevention in the age of rising antibiotic resistance and the necessity for bio-based treatment options.However, there are some significant gaps in the literature:

- Lack of direct in vitro investigation of alfalfa honey on human skin cell lines (e.g., HaCaT keratinocytes): No study to date has evaluated its cytotoxicity, effects on proliferation, or wound closure on skin cells.

- Inadequate comparison studies of alfalfa honey with other monofloral honeys using standardized experimental conditions.

- Lack of functional assays bridging its antioxidant content and biological activity in skin models.

2.7.3 Avocado Honey

2.7.3.1 Origin and Composition

Avocado honey is a monofloral honey gathered from the nectar of Persea americana (avocado) flowers. It is predominantly manufactured in nations such as Central and South America, Spain, and some parts of the Mediterranean. (Can-Alonzo, et al., 2005). Avocado honey is dark amber in color, with a heavy texture and strong, molasses-like flavor. Like the majority of honeys, its primary constituents are simple sugars, mainly glucose and fructose, which make up about 70–80% of its content.

Notably enough, studies have proved that avocado nectar is relatively less attractive to honey bees compared to other floral sources. This reduced appeal may be due to some of the nectar's properties, such as taste, odor, or composition. (Afik, Dag, & Shafir, 2006). While the reduced pollinator attraction towards avocado nectar, it still has sufficient bee activity to support honey production, though at lower frequencies than with more attractive nectars. The acidic pH and phytochemically of the honey are responsible for its measured antioxidant and antimicrobial activity.

It is documented that avocado honey has a variety of phenolic acids, which may account for antioxidant and antimicrobial properties. Reports show that by-products of avocados, namely the peel and seed, have similar phenolic constituents to honey, including protocatechuic acid, caffeic acid, sinapic acid, and/or hydroxybenzoic acids (Araújo et al., 2023; Yahia et al., 2022). These agencies are involved in free radical scavenging, lipid peroxidation involvement, and modulation of inflammation, which might increase the healing properties of avocado honey for wound treatments (Lyu, Agar, Barrow, Dunshea, & Suleria, 2023).

Besides phenolic acids, an interesting profile of flavonoids has been detected in avocado honey, that results mainly responsible for its strong antioxidant and anti-inflammatory effects. The most often reported flavonoids are epicatechin, a bioactive

flavanol characterized by antioxidant properties, and kaempferol, a bioactive flavonol that has been demonstrated to have anti-inflammatory and organ-protective effects (Koemel, et al., 2023). These flavonoids, like other minor flavonols and flavanols, participate synergistically in the total bioactivity of avocado honey and validate its functional therapeutic capacity.

2.7.3.2 Ethnomedicinal and Clinical Relevance

Although avocado honey is less documented in traditional medicine, Avocado was long valued for its emollient and healing properties in the skin. Clinical evidence supporting avocado honey application in wound healing or skin regeneration is nonexistent. While avocado honey had high antimicrobial and antioxidant activity in experiments, its action in human skin cells or wound healing in clinical trials has never been studied

2.7.3.3 Insights from In Vitro Cellular Studies

There is no reviewed in vitro study that has explicitly tested the effect of avocado honey on human skin cells like keratinocytes or fibroblasts to date. However, meaningful data can be inferred from studies that have analyzed extracts of other parts of the avocado tree, precisely the seed and fruit. Such studies offer a pre-existing awareness of avocado's intrinsic bioactivity at the cellular level and suggest possible avocado honey therapeutic applications to explore.

Researchers in one study experimented with different avocado seed extracts on human skin cells. They found that certain extracts made the keratinocytes (skin cells) grow and stay healthy, and activated important genes in skin repair, like EGF and KGF receptors. This would mean the extracts can heal the skin. For fibroblasts, cells which help in the formation of new tissue, some of the extracts exhibited mildly enhanced growth, while others had less effect.

Due to its antioxidant and anti-inflammatory composition, it is logical to expect avocado honey to facilitate cell migration and reduce oxidative stress in wound conditions.

Its effectiveness should be confirmed in subsequent research based on standardized cellular models.

2.7.3.4 Antimicrobial Activity

Avocado honey exhibits strong antimicrobial activity, particularly against wound-associated pathogens. It is due to a variety of reasons, such as low pH, high sugar content, the generation of hydrogen peroxide, and high phenol content. Studies that evaluated the antimicrobial properties of dark honeys have shown inhibition of Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa, among others (Alvarez-Suarez et al., 2010).

In a recent comparative study on avocado, eucalyptus, and rapeseed honeys, it was found that avocado honey at a 20% w/v concentration was most effective in preventing biofilm formation as well as in degrading already established biofilms. This was tested with two clinically significant pathogenic bacteria: Staphylococcus aureus (Gram-positive) and Klebsiella pneumoniae (Gram-negative), both of which are known to complicate wound healing and are prone to resist conventional therapy (Combarros-Fuertes P. , et al., 2020).

Avocado honey, polyphenolic-rich and low in water content, is likely to exhibit similar inhibitory activities. These might be hydrogen peroxide, organic acids, and polyphenols, which may act synergistically. (Combarros-Fuertes P. , et al., 2020). particularly in biofilm prevention. In addition to being bacteriostatic (growth-inhibiting) in character, avocado honey was also bactericidal, where the minimum lethal concentration (MLC) values closely approached the minimum inhibitory concentrations (MICs). It indicates that not only does the honey inhibit the growth of bacteria, but at slightly higher concentrations, it can kill bacteria.

2.7.3.5 Antioxidant Potential and Phytochemical Content

Avocado honey is rich in antioxidants like phenolic acids and flavonoids that give it the dark colour and strong antioxidant activity. Comparative antioxidant test assays such as

DPPH and FRAP, have indicated that the darker honeys, such as avocado, are likely to exhibit more radical-scavenging activity than the lighter honeys.

One of the experiments analyzed several monofloral honeys using three spectrophotometric assays for the estimation of antioxidant activity. The avocado had the highest total phenolic content (TPC) and total flavonoid content (TFC) among the samples. It was also best in the reducing power (RP) test, which measures the ability of antioxidants to donate electrons and neutralize free radicals. This suggests that phenolic acids from avocado honey are responsible for the potential of antioxidants. This is in line with the proposed utility of avocado honey as a functional natural food with antioxidant health benefits (Combarros-Fuertes P. , et al., 2018).

Additionally, some of the avocado extracts had high antioxidant activity, which would protect cells from harm. While these results are from the avocado plant and not the honey, they suggest that avocado honey might also be beneficial to skin cell health, and more research is needed to determine if so (Ramos-Jerz, Villanueva, & Deters, 2007).

2.7.3.6 Summary and Knowledge Gaps

Avocado honey demonstrates promising biological activity, positioning it as a potentially valuable therapeutic agent in wound care and general health. Its biochemical make-up provides the basis for antimicrobial, antioxidant, and potentially wound-healing activity. While direct clinical or in vitro evidence is currently sparse, extrapolating from its biochemical profile and comparing it with other honey varieties suggests that it has the potential to aid tissue regeneration, reduce inflammation, and prevent microbial growth in wound environments.

Nonetheless, existing evidence shows that avocado honey has the potential as a valuable natural product in integrative wound care options and antimicrobial therapy. Some significant gaps in the literature include:

- The absence of direct in vitro studies evaluating the effect of avocado honey on human skin cells and macrophage behavior, including cell viability, proliferation, migration, and inflammatory response.

- Clarifying the phenolic profile and antioxidant activity of avocado honey and its relationship with cellular effects.

- Lack of clinical trials to establish its therapeutic efficacy and safety in human wound care.

2.8 Manuka Honey (Reference benchmark)

2.8.1 Origin and Composition

Manuka, from New Zealand's native plant Leptospermum scoparium, is biochemically diverse, which means it is very rich in chemicals, giving it its famous healing properties.These phenolic acids include gallic acid, caffeic acid, p-coumaric acid, syringic acid, and chlorogenic acid, which are the main factors behind its antioxidant and anti-inflammatory properties (Wang H. , 2011).The honey also harbors a wide variety of significant flavonoids, including pinobanksin, pinocembrin, chrysin, luteolin, quercetin, and kaempferol, which have a dual effect as antioxidants, anti-inflammatories, and fighting against microorganisms (Morar, et al., 2025)

It contains bioactive molecules that make it unique compared with other kinds of honey besides classical polyphenols. Such compounds as methylglyoxal (MGO) as the main Mānuka non-peroxide antibacterial factor, leptosperin, a glycoside of methyl syringate (a floral marker), methyl sulfoxides, and 2′-methoxyacetophenone are found in relatively high quantities in this honey, which contribute to determining its specific chemical profile and antimicrobial power (Adams C. J., et al., 2008). The presence of these phenolic acids, flavonoids, and specific compounds is responsible for the wound healing potential, antioxidant properties, and antimicrobial activity in Manuka honey.

beside the Unique Manuka Factor (UMF). Besides MGO, manuka honey also contains hydrogen peroxide, low pH value (in general 3.5 and 4.5), and phenolic compounds, which all contribute to its medicinally effective outcomes.

It also contains high levels of bee defensin-1 as well as trace levels of other compounds that make additional contributions to its healing and antimicrobial properties. manuka honey contains phenolic compounds such as methyl syringate, leptosin, and other flavonoids that contribute to its strong antioxidant and immunomodulatory activities. The UMF label ensures that the honey has undergone testing of three signature markers for leptosperin, dihydroxyacetone (DHA), and MGO (Adams C. J., et al., 2008).

2.8.2 Clinical Applications in Wound Management

Manuka honey is widely known and clinically used for wound healing, particularly the healing of infected chronic wounds. Its healing activity is a consequence of its multi-component mechanisms of activity; it maintains the wound bed in a moist state, forms a protective barrier, and exerts antibacterial and immunomodulatory activities (Cooper, Molan, Krishnamoorthy, & Harding, 2001). Manuka honey is highly recommended in clinical treatments where drug-resistant infections are an issue. additionally, burns, diabetic foot ulcers, pressure ulcers, and chronic ulcers, because higher UMF values, typically 10+ or above, reflect strong and effective antimicrobial qualities. (Adams C. J., et al., 2008) .

Particular emphasis has been placed on manuka honey's ability to combat multidrug-resistant bacteria. It was found to eliminate methicillin-resistant *Staphylococcus aureus* (MRSA) , (Jenkins, Cooper, & Burton, 2011). from infected wounds and restore bacterial susceptibility to conventional antibiotics like oxacillin, Pseudomonas aeruginosa, which frequently result in chronic wounds (Petrović, Lučić, Radovanović, & Martić, 2024).

Its acidic nature also stimulates macrophage bactericidal action and increased fibroblast activity in chronic wounds. Its growth factors, like TGF-β, are also stimulated in these acidic conditions, stimulating increased cellular function, as seen in human dermal fibroblast (hDF) experiments as well as in in vitro models of wound closure (Jull, Jenkins, Cooper, & Burton, 2011).

Recent research laboratory work has also established manuka honey's capacity to heal wounds. In a research, scientists studied the effect of manuka honey on fibroblasts and keratinocytes from patients suffering from chronic otitis media. The cells were treated with low concentrations of honey (0.04%, 0.1%, and 0.25%) and cultured for a period of time.

Findings showed that manuka honey promoted keratinocytes to multiply more than in the control cells, especially at low concentrations. Although the fibroblast count was not higher, the doubling time was shorter, hence they increased more rapidly. (Priyono, et al., In vitro activity of Manuka and Trigona honey on fibroblast and keratinocyte cultures, 2024) Honey also increased secretion of keratinocyte growth factor (KGF), the growth factor that stimulates skin repair. All these supports the fact that manuka honey can increase cell growth and wound healing, and is a great reference control for studies like mine.

2.8.3 Antioxidant and Anti-inflammatory Properties

Manuka honey is known for its antioxidant power. These beneficial properties are mainly due to its high content of phenolic compounds such as hydroxybenzoic acid and methyl syringate. Manuka honey has recently been reported to have strong antioxidant activity, which is greater than that of other types of honey. For example, the 2025 study reported that Manuka honey had a significantly higher phenolic content than Ohia Lehua honey and that this corresponded with increased antioxidant capacity (Morar, et al., 2025).

The antioxidant properties of Manuka honey have been assessed through several assays, such as Ferric Reducing Antioxidant Power (FRAP), DPPH radical scavenging activity, and hydrogen peroxide scavenging capacity. Consistent with these techniques, it has been reported that Manuka honey can neutralize free radicals and limit oxidative stress,

which is important to prevent the cellular damage linked with chronic diseases such as cardiovascular diseases, diabetes mellitus, and neurodegenerative pathologies (Chau, Owusu-Apenten, & Nigam, 2017).

A review additionally suggested that Manuka honey has antioxidant power similar to high-quality honeydews. The review concluded that it's useful in treating oxidative stress (Grabek-Lejko, Miłek, & Dżugan, 2024).

2.8.4 Anti-inflammatory Properties

Manuka honey is also well known for its efficacy in reducing inflammation, and this feature has been exploited through clinical applications in the treatment of topical or systemic diseases. Recent studies suggest that such effects of honey are partially due to its ability to modify the inflammation-related pathways and cytokine production.

In 2004, another study reported that unprocessed Manuka honey inhibited TNF-α production by rat neutrophils at the concentration of 400 μg/mL (31). Interestingly, at the lower concentration (100 μg/mL), this honey induced TNF-α secretion, indicating a dose dependency of the effects that may play a role in its anti-inflammatory potential (Iosageanu A. , Stefan, Craciunescu, & Cimpean, 2024).

Further, an overview in 2025 mentioned that honey (Manuka or other) exerts anti-inflammatory effects by inhibition of pro-inflammatory cytokines levels such as TNF-α, IL-1β, and IL-6. This modulation means that Manuka honey can alleviate some of the inflammation response present in many chronic diseases (Saad, 2025).

Additionally, in a model of embryonic kidney cells and ulcerative colitis, manuka honey inhibited inflammatory responses via the TLR1/TLR2 pathway. Such pathway-directed anti-inflammatory activities further reemphasize its therapeutic significance towards the cure of chronic inflammatory diseases**.** Furthermore, by regulating pro-inflammatory

cytokines like TNF-α and IL-1β, manuka honey reduces tissue damage and encourages healing (Majtan, Methylglyoxal—A potential risk factor of manuka honey in healing of diabetic ulcers, 2011)

2.8 Comparative Summary of Reviewed Honeys

The honey types explored in our study, Citrus, Alfalfa, Avocado, and Manuka, are different in their botanical origins, chemical compositions, and biological activities relevant to wound healing. Table 2.4 presents a comparative overview of these honeys across various key parameters, drawing from existing literature.

Table 2.4: Physicochemical and Traditional Features of Selected Honeys

Parameter	**Citrus Honey**	**Alfalfa Honey**	**Avocado Honey**	**Manuka Honey**
Botanical Source	Citrus spp. (e.g., orange, lemon)	Medicago sativa (alfalfa)	Persea americana (avocado)	Leptospermum scoparium
Color / Appearance	Light amber, clear	Amber	Dark amber, thick	Golden
Phenolic Content	low total phenolic content, flavonoids (hesperetin, hesperidin, rutin), phenolic acids (caffeic, p-coumaric, syringic)	Moderate: phenolic acids (caffeic, p-coumaric, caftaric, chlorogenic), flavonoids (kaempferol, quercetin)	High: phenolic acids (caffeic, protocatechuic, ferulic), flavonoids (epicatechin, kaempferol)	Very high: phenolic acids (gallic, caffeic, p-coumaric), flavonoids (pinobanksin, pinocembrin, chrysin, luteolin, quercetin, kaempferol),

				MGO, leptosperin
Traditional Medicinal Use	Widely used in Palestine for skin and digestive conditions	Folk medicine: immunity, digestion	Limited use, mostly topical	Extensively documented in Māori and modern medicine

Of honeys examined, Manuka honey always serves as the gold standard of evidence from clinical trials, antibacterial action against a broad spectrum of bacteria, and antioxidant activity largely due to its unique methylglyoxal (MGO) content. It has wide-ranging uses for modern wound care and possesses FDA-approved medical indications.

On the other hand, Palestinian honeys such as Citrus, Avocado, and Alfalfa have very encouraging in vitro bioactivities, including antimicrobial and antioxidant activity, but have yet to be fully exploited in wound-healing use. Citrus extract and Avocado honeys show robust antioxidant and cell migration properties, suggesting equal potential to other dark polyphenol honeys. Alfalfa honey, despite traditional use, has been the least studied regarding keratinocyte response and regeneration outcomes.

Cumulatively, several knowledge gaps persist among these honeys: limited in vitro studies with standardized human keratinocyte models, no evaluation of oxidative stress and inflammation pathways, and a complete lack of clinical trials outside of Manuka. These gaps and differences in the literature are highlighted in Table 2.5

Table 2.5: Biological and Clinical Evidence of Selected Honeys

Parameter	**Citrus Honey**	**Alfalfa Honey**	**Avocado Honey**	**Manuka Honey**
Antioxidant Capacity	Moderate (DPPH, FRAP)	Moderate, limited data	High (DPPH, FRAP, RP)	Highest (TPC, TFC, MGO)

Antibacterial Activity	Effective against Gram-positive bacteria	Mild antibacterial, inhibits biofilms (e.g., Acinetobacter baumannii)	Strong vs. S. aureus, K. pneumoniae, prevents & disrupts biofilms	Broad-spectrum including MDR strains (e.g., MRSA, P. aeruginosa)
In Vitro Cellular Effects on Keratinocytes	Enhances keratinocyte viability, moderate wound closure	Fibroblast proliferation, collagen deposition; limited direct keratinocyte data	Suggests enhanced keratinocyte migration & antioxidant defense (from extracts not directly from honey)	Promotes proliferation, migration, and modulates cytokine pathways
Documented Clinical use on skin wounds	Minimal clinical trials	Sparse evidence	No known clinical trials	FDA-approved Comvita®, widely used in chronic wound care
Cytotoxicity on Keratinocytes	Not studied	Not studied	Not studied	Yes, dose-dependent
Cell Migration (Scratch Assay)	Not studied	Weak evidence	Moderate evidence (only from extracts)	Documented in HaCaT models

Addressing these gaps will be essential in validating Palestinian honeys as a therapeutic potential for evidence-based wound care. This kind of finding underlies the rationale of this

study, which is to explore and compare the wound healing activity of Citrus, Avocado, and Alfalfa honeys on HaCaT cells under in vitro conditions.

2.9 Conclusion of Literature Review

While the wound healing potential of honey has been recognized since ancient times, the bioactive properties of honey have caused wound healing via antimicrobial activity, antioxidant defense, anti-inflammatory modulation, and induction of proliferation, angiogenesis, cell migration, and extracellular matrix formation. Among these, Manuka honey has been studied most extensively, and there is strong evidence for its clinical use in chronic wounds and burns. Its proprietary MGO (methylglyoxal) based activity is the proudest for both the lab and clinic.

In contrast, Palestinian honeys like Citrus, Avocado, and Alfalfa are exhibiting favorable antioxidant and antibacterial effects in terms of their polyphenols and vitamin content. First in vitro studies show potential for cell migration and protection from oxidative stress; however, no data are yet available on standardized human keratinocyte models. Alfalfa nectar has significant traditional use, and wound healing studies of this honey are particularly scarce; there is minimal evidence for its cellular effects. Furthermore, no human studies on the therapeutic usage of the honeys in human wounds have been done so far.

Taken together, therefore, the literature indicates a lack of systematic in vitro studies on models of human keratinocytes or other cell types and Palestinian honey related to the biological rationale supporting the use of Palestinian honeys in wound treatment. Bridging this gap will be crucial for the characterization of their potential in wound healing and to promote additional translational research.

The subsequent chapter has thus been focused on the experimental strategy that we have used to establish and compare the wound-healing abilities of Citrus, Avocado, and Alfalfa honeys towards HaCaT keratinocytes by analyzing their capacity to affect cell viability, proliferation, migration, as well as modulating oxidative stress in these cells.

3 Chapter Three: Methodology

Based on previous literature and the use of honeys in folk medicine, the present study aims to investigate the influence of three Palestinian honeys, citrus, alfalfa, and avocado, on human skin cells during the processes of wound healing. Including cell viability, proliferation, migration, and inflammation, using the HaCaT keratinocyte cell line as an in vitro model.

Different assays corresponding to different stages of wound healing were employed. MTT assay measured cell metabolic activity as an indirect index of viability and proliferation, whereas cytostatic effect refers to inhibition of proliferation without significant cytotoxicity, assessed by prolonged incubation. Scratch wound-healing assay confirmed honey's impact on keratinocyte migration under conditions that simulate the re-epithelialization process. Nitric oxide (NO) content was quantified using the Griess assay in order to investigate anti-inflammatory potential and regulation of the inflammatory phase of healing.

In addition, external assays were performed to supplement in vitro wound healing: antimicrobial assays examined honey's antibacterial activity against clinically important bacterial isolates, and molecular docking simulations of putative honey polyphenolic compounds interacting with primary target proteins predicted the likely interactions of honey polyphenolic compounds with primary target proteins to explore their mechanistic basis for bioactivity.

By comparing these assays, the study presents a general in vitro evaluation of the therapeutic potential of Palestinian honeys compared with Manuka honey as a benchmark. It would be anticipated that the Palestinian honeys will enhance keratinocyte proliferation and migration and modulate inflammation, giving insight into their therapeutic value in wound care and a rationale to explore the molecular mechanisms involved in wound healing by honey.

3.1 Study Design

This study was an experimental in vitro research project to assess the prospective ability of selected types of Palestinian honeys to heal wounds, using the immortalized human keratinocyte (HaCaT) cell line as a biological model.

The study employed a comparative strategy to examine three types of Palestinian honeys (avocado, alfalfa, and citrus) and Manuka honey as a reference control, given its established medicinal use in wound care. Various concentrations of honey samples were diluted and incubated with HaCaT cells according to standardized conditions.

A selection of functional assays was employed to represent different events of the wound healing cascade. Cell viability and proliferation were assessed by the MTT assay, and cell migration and wound closure were assessed by the scratch wound-healing assay. For inflammatory modulation, nitric oxide production was assessed by the Griess assay.

The multi-assay protocol allowed phase-specific quantification of honey effects in relation to the inflammatory, proliferative, and migratory phases of wound healing. In collaboration with external researchers for antimicrobial and molecular docking studies on selected honeys. These results, adding to our study findings, will be discussed and compared in Chapter Five. The design of the study is summarized in Figure 3.1

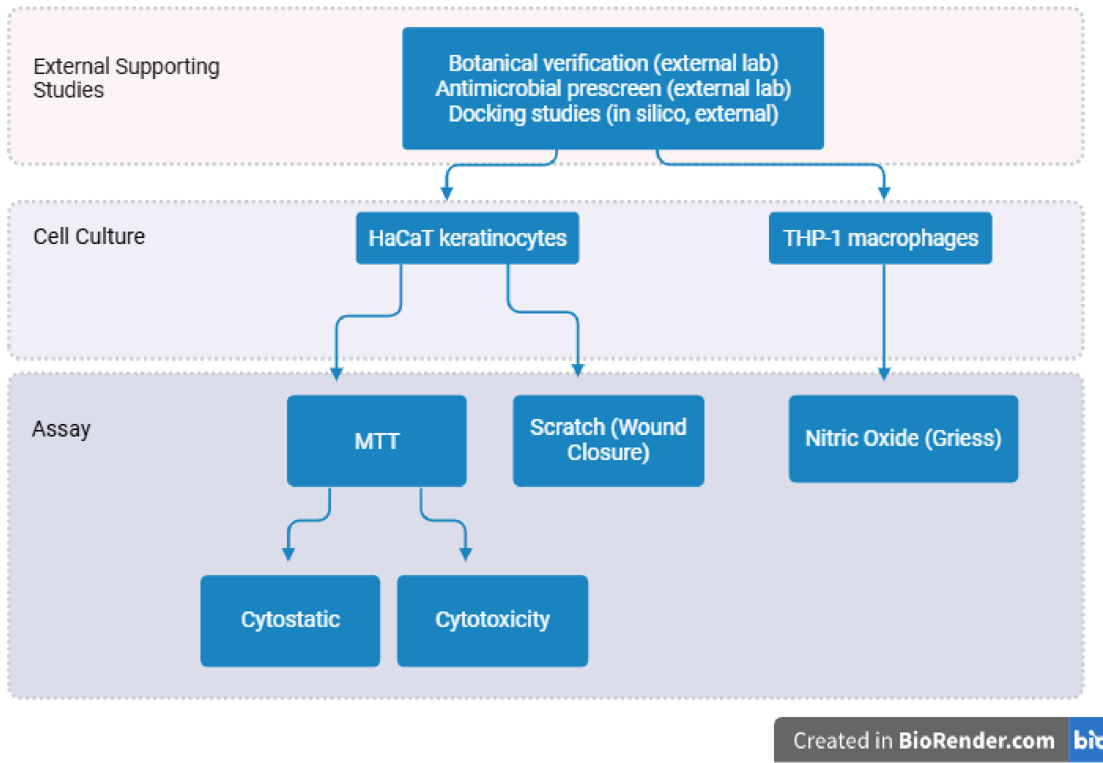

Figure 3.1: Study flowchart, sample handling to cell culture, then experimental assays

The study design ensured replicability by allowing triplicate samples per condition, the addition of negative controls (uninoculated cells), and the comparison with the manuka honey. These data were statistically evaluated to determine significant differences between honeys and concentrations, thereby determining both the biological relevance and therapeutic value of the Palestinian honeys for wound healing.

3.2 Materials and Reagents

1. Cell Lines

- HaCaT human keratinocyte cell line (CLS, Cat# 300493, Germany)
- THP-monocytic leukemia cell line (ATCC TIB-202, USA)]

2. Media and Supplements

- RPMI-1640 Medium (Gibco, USA)

- DMEM (Biological Industries, Israel)
- Fetal bovine serum (FBS), heat-inactivated at 56°C for 30 minutes
- Non-essential amino acids (Gibco, USA)
- L-glutamine (Gibco, USA)
- Penicillin-Streptomycin solution (Gibco, USA)
- Phosphate-buffered saline (PBS) (pH 7.4)

3. Honey Samples

- Citrus, Alfalfa, Avocado honeys (Naba Al-Asal, Tulkarem, northern West Bank, Palestine)
- The fourth honey, Manuka honey from Comvita®, a certified and internationally recognized New Zealand-based

4. MTT Assay Reagents and Solutions

- MTT reagent (5 mg/mL in PBS), Sigma-Aldrich, stored at 4°C protected from light.
- Solubilization solution: 100 µL per well of a solution comprising 90% isopropanol and 10% formic acid used to dissolve formazan crystals post-incubation.
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) used alternatively for formazan dissolution if required.
- Sterile 96-well flat-bottom tissue culture plates (Corning, USA).

5. Wound Scratch Assay Materials

- Sterile 24-well plates for cell culture.
- Sterile 200 µL pipette tips to create scratches.
- Trypsin-EDTA (0.25%) solution for cell detachment.

6. Griess Assay Reagents

- Sulfanilamide (Fluka AG, No. 86060)
- N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) (Sigma, N-9125)
- Phosphoric acid (H_3PO_4) (Fluka AG)
- Reagent A: 0.1% (w/v) N-(1-Naphthyl) ethylenediamine dihydrochloride (NED) (Sigma, N-9125) dissolved in distilled water; stored at 4°C and used within one week

- Reagent B: A mixture of 1% (w/v) sulfanilamide and 3% (v/v) phosphoric acid prepared by dissolving 500 mg sulfanilamide and adding 1.5 mL phosphoric acid to a final volume of 50 mL with distilled water; stored at 4°C.
- Sodium nitrite ($NaNO_2$) analytical standard (Sigma-Aldrich) used for preparation of standard curves (0–100 µM).

7. Consumables and Equipment

- Sterile pipette tips, centrifuge tubes, culture flasks.
- Hemocytometer or automated cell counter.
- Biosafety cabinet Class II for aseptic work.
- Microplate reader capable of reading at 570 nm (MTT) and 540–540 nm (Griess)CO_2 incubator (37°C, 5% CO_2).
- Refrigerated centrifuge.
- Water bath (37°C).

3.3 Ethical Considerations

This study was conducted within an in vitro model, the human keratinocyte cell line HaCaT and the human monocytic cell line THP-1. Since no human subjects, animal models, or clinical specimens were employed, formal approval by a medical ethics committee or institutional review board (IRB) was not required. Nevertheless, the research design complied with national and international standards. All cell culture procedures were performed under aseptic conditions in a biosafety level II laboratory.

Cells were handled only in a laminar flow under a biosafety cabinet that is certified to maintain asepsis and researcher safety. Additionally, standard cell thawing, passaging, sustaining, and disposal procedures were followed. Waste materials and chemical reagents were disposed of in accordance with institutional biosafety and hazardous waste disposal procedures to prevent environmental harm or contamination. In addition to biosafety adherence, the studies were conducted following Good Laboratory Practice (GLP), scientific

integrity, and transparency. The reagents and cell lines were purchased from established suppliers, and experimental protocols were well documented to ensure reproducibility.

3.4 Honey Sample Collection and Botanical Characterization

Four types of monofloral honey were used in this study: citrus, avocado, alfalfa, and Manuka. The citrus, avocado, and alfalfa honeys were sourced locally from the same agroecological region to ensure consistency of climate, soil, and foraging conditions to minimize environmental variability. Additionally, the study focuses on Palestinian honeys; the local samples were obtained from Naba Al-Asal, a well-known producer of honey located in Tulkarem, northern West Bank, Palestine.

Each honey was harvested during its respective blossoming time in 2021 or 2022 and placed directly into sealed food-grade jars as soon as it was harvested to prevent contamination, oxidation, or undesired premature fermentation. The storage duration is approximately 2-3 years. The local samples were stored in a dry, cool, and clean location at room temperature until experimentation. The botanical origin of all honeys was confirmed based on the records of the beekeepers. Table 3.1 recapitulates the botanical, geographical, and temporal data per local honey sample used throughout the study.

Table 3.1: Honey samples IDs and their botanical, geographic origins, and harvest year.

English name	Scientific name	Location	Year of harvest
Alfalfa	*Medicago sativa*	Jenin	2021
Avocado	*Persea americana*	Tulkarm	2021
Citrus	*Citrus limon*	Tulkarm	2022

The fourth honey is the commercial Manuka (*L. scoparium*) (Comvita®, Vaughan, ON, Canada), obtained directly from Comvita—a New Zealand-based company that is registered and internationally accredited for producing medical-grade, high-quality honeys. Comvita® is known for its 'pipeline' supply chain ethics to assure quality, standardized processing, and independently testing each batch for authenticity and potency (Last: Nicholls & Newlands, 2004).

The sample selected was rated UMF +15 (with a methylglyoxal (MGO) equivalent of 514+ mg/kg) – both indicative of robust antibacterial activity and quality. The UMF grading system is an internationally recognized scale of rating honey's non-peroxide activity, i.e., the level of naturally present bioactive compounds, such as methylglyoxal (MGO), DHA, and the role with leptosperin markers, making it applicable in scientific and clinical markets for research reproduction (Wang, Qiu, & Zhu, 2024).

This brand was selected due to its published reliability, global regulatory acceptance, and prior extensive use in peer-reviewed biomedical research as a positive control in comparison laboratory experiments. The extensive product documentation, batch traceability, and storage recommendations also make it appropriate for controlled in vitro testing. This use of a commercially available certified Manuka honey provided excellent support for comparison between biological and therapeutic properties.

The method not only facilitated comparative benchmarking under identical experimental conditions but also provided a standard reference to demonstrate the impact of regional honeys on keratinocyte skin cells and antimicrobial capacity compared to an independently certified international medical-grade standard. Manuka honey was kept at room temperature in its original sealed container with the local samples to ensure the stability of bioactivity until testing. The colors of honey samples are respectively presented in Fig. 3.2.

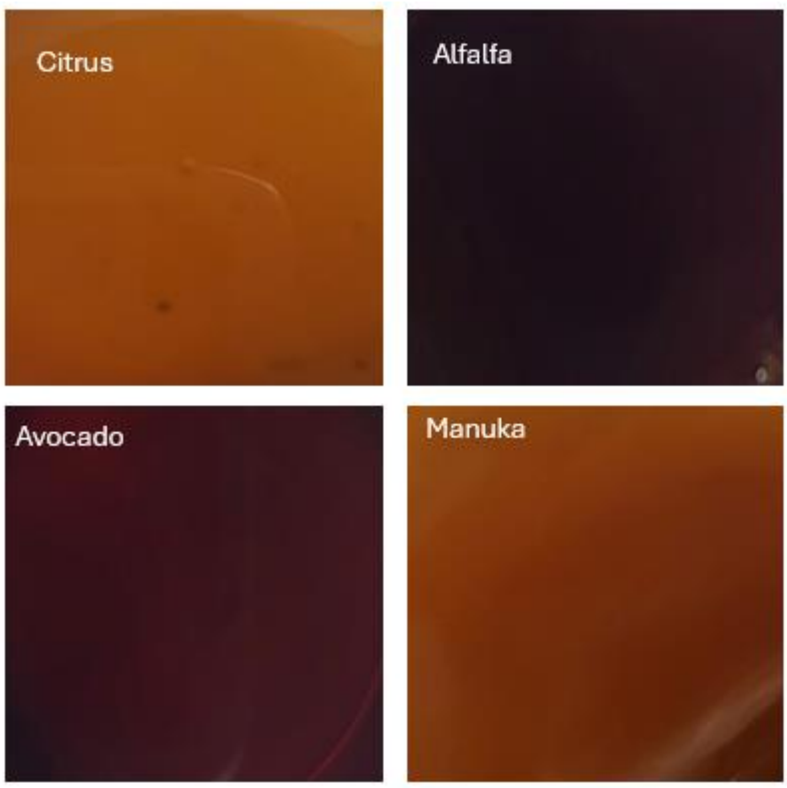

Figure 3.2: Visual representation of the Citrus, Alfalfa, Avocado, and Manuka honey samples showing their color differences.

3.5 Cell Culture Protocol

3.5.1 Cell Lines and Culture Conditions

This study used two human cell lines. Although the study focused more on keratinocytes, represented by HaCaT, THP-1 monocytic cells were also used to assay the inflammatory response by nitric oxide production following macrophage differentiation. Both of these cell lines are well-established in in vitro wound healing and inflammation research, respectively. As detailed in Chapter 2 (the literature review), both cell lines were cultured under optimized and sterile conditions, including mycoplasma testing to ensure viability, reproducibility, and biological relevance to the models under investigation. Mycoplasma screening was performed using a standardized detection assay. Their culture

conditions were strictly optimized to afford cellular responsiveness and experimental reproducibility across assays.

3.5.1.1 HaCaT Cells

HaCaT is a spontaneously immortalized human keratinocyte cell line that is routinely utilized as a model to investigate skin regeneration and epithelial wound healing. Cells were cultured in RPMI-1640 medium (Roswell Park Memorial Institute Medium; Gibco, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) that supplies the required hormones, nutrients, and growth factors. To promote the best cell function and growth, the medium was further supplemented with 1% non-essential amino acids (NEAA), 1% L-glutamine, 2,100 U/mL penicillin, and 10 µg/mL streptomycin.

The cells were grown in T-75 cm^2 culture flasks at 37°C in a humidified 5% CO_2 incubator. Additionally, the medium was replaced every 48–72 hours to sustain metabolic activity and remove waste products.

3.5.1.2 THP-1 Cells

THP-1 is a human monocytic leukemia cell line derived from an acute monocytic leukemia patient (ATCC TIB-202). The cells were maintained in DMEM (Biological Industries, Israel), with the addition of 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cultures were maintained in suspension in T-25 or T-75 flasks, at 37 °C, 5% CO_2, and the cell density was $0.2–1 \times 10^6$ cells/mL. Only healthy log-phase cells were used for differentiation and later assays.

3.5.2 Subculturing and Expansion Procedures:

The cell culture medium was replaced every 48 to 72 hours to deliver fresh nutrients and remove metabolic waste. HaCaT cells were subcultured once they were at around 70–80% confluency to prevent overcrowding and ensure healthy proliferation. Cells were

washed in sterile phosphate-buffered saline (PBS) to remove serum proteins and loosened using 0.25% trypsin-EDTA solution (Gibco). Microscopic observation was performed after trypsinization for 7–9 minutes for HaCaT, and once the cells got detached thoroughly, the reaction was arrested using complete medium with serum supplementation. THP-1 cells grow as suspension cells; subcultured by dilution to keep densities ~2×10^5–5×10^5 cells/mL. cells/mL. Suspension was centrifuged at 1000 rpm for 5 minutes, and the resulting pellet was re-seeded and re-suspended to the appropriate density depending on the desired downstream application.

3.5.3 Cryopreservation and Recovery of Cells:

HaCaT and THP-1 cells were cryopreserved in freezing media containing 90% FBS and 10% DMSO to keep early-passage cells and minimize phenotypic drift. Aliquots of the cell suspensions were slowly frozen to a cooling rate of -1°C per minute in a freezing container and then stored for prolonged periods in liquid nitrogen.

Thawing was rapidly performed in a 37°C water bath, followed by dilution immediately in pre-warmed complete medium to reduce the toxicity of DMSO. Cells were spun down to remove any remaining DMSO and transferred into fresh culture medium, and placed in fresh flasks. Only cells between passages 5–15 were used in all experimental assays for genetic stability, as well as to rule out culture-related abnormalities.

3.5.4 THP-1 Differentiation and Stimulation Protocols

For inducing macrophage-like differentiation, THP-1 cells were treated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and seeded in 96-well flat-bottom plates. After 24–48 hours, the cells that were attached to the plate had attained morphological and functional features of macrophages. PMA-supplemented medium was replaced by fresh medium free from PMA and incubated for another 24 hours. The cells were then treated with 1 µg/mL lipopolysaccharide (LPS) to mimic inflammatory circumstances and induce inducible nitric oxide synthase (iNOS) production. After activation, cells were treated with honey of varying

concentrations to determine anti-inflammatory activity with nitric oxide production as readout in an assay.

3.5.5 Honey Treatment Setup and Experimental Conditions

Cells were seeded and then incubated with the test samples in honey-treated groups (in case of HaCaT) or after LPS stimulation (in case of THP-1).

Controls and Replication

For validation of results validity and reproducibility:

- Negative controls (medium alone)
- Positive control experiments (LPS alone in THP-1 or manuka in HaCaT) were also maintained to ensure experimental responses.

All treatment groups were performed in triplicate in biological and technical replicates within a plate to increase reproducibility and statistical power.

3.5.6 Cell Counting, Viability Test, and Seeding

For all experiments, viable cells were counted using a hemocytometer and the trypan blue exclusion method. After detachment with trypsin-EDTA, an aliquot of the cell suspension was mixed 1:1 with 0.4% trypan blue solution and loaded onto a hemocytometer. Only viable (unstained) cells were counted. The cell concentration was calculated and adjusted using fresh complete medium to reach the desired seeding density. For MTT cytotoxicity assays, HaCaT cells were seeded at 20,000 cells/well in 96-well plates. For cytostatic and proliferation assays, 5,000 cells/well were seeded in a way that allowed longer-term monitoring of cellular growth. In the nitric oxide assay, PMA-differentiated THP-1 macrophages were seeded at 50,000 cells/well before treatment. All the plates were incubated under identical conditions and handled simultaneously to minimize variation.

3.5.7 Plate Layout Diagrams

Each 96-well plate was pre-labeled with a plate map for observation of conditions. Plate layouts for the MTT assay for cytostatic and cytotoxic are the same, which is:

- Rows for concentrations (mg/ml): row A: Control, row B: 2mg/ml, row C: 4mg/ml, row D: 8mg/ml, row E: 16 mg/ml, Row F-H: Blank.
- Columns for Honey types: Column 1-3: Citrus, Column 4-6: Alfalfa, Column 7-9: Avocado, Column 10-12: Manuka

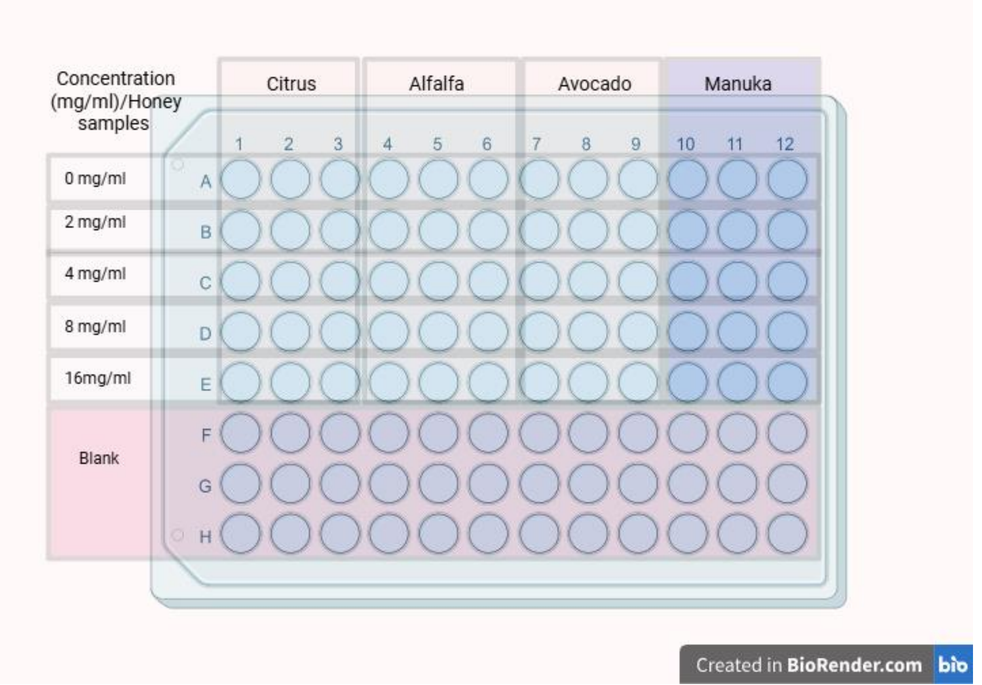

Figure 3.3: 96-well plate layout for MTT assays, cytotoxic, and cytostatic.

As for the Wound scratch assay, two 24-well plates were pre-labeled with a plate map for observation of conditions like this:

- Rows for concentrations (mg/ml): row A: Control, row B: 1mg/ml, row C: 2mg/ml, row D: 4mg/ml.

- Columns for Honey types: Column 1-2: Citrus, Column 3-4: Alfalfa, Column 5-6: Avocado.

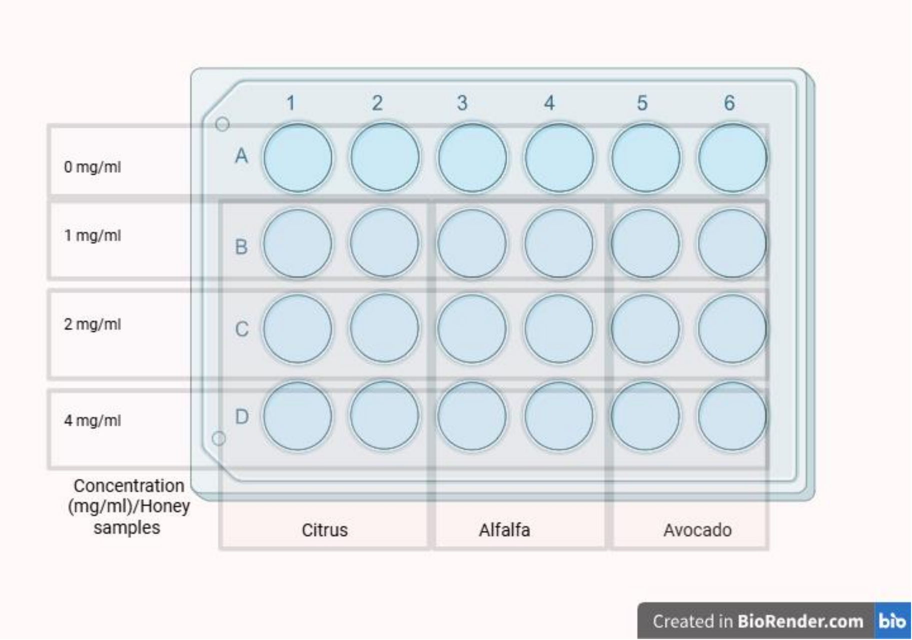

Figure 3.4: first 24-well plate layout for wound scratch assay.

As for the second well plate:

- Rows for concentrations (mg/ml): row A: Control, row B: 1mg/ml, row C: 2mg/ml, row D: 4mg/ml.
- Columns for Honey types: Column 1-2: Manuka.

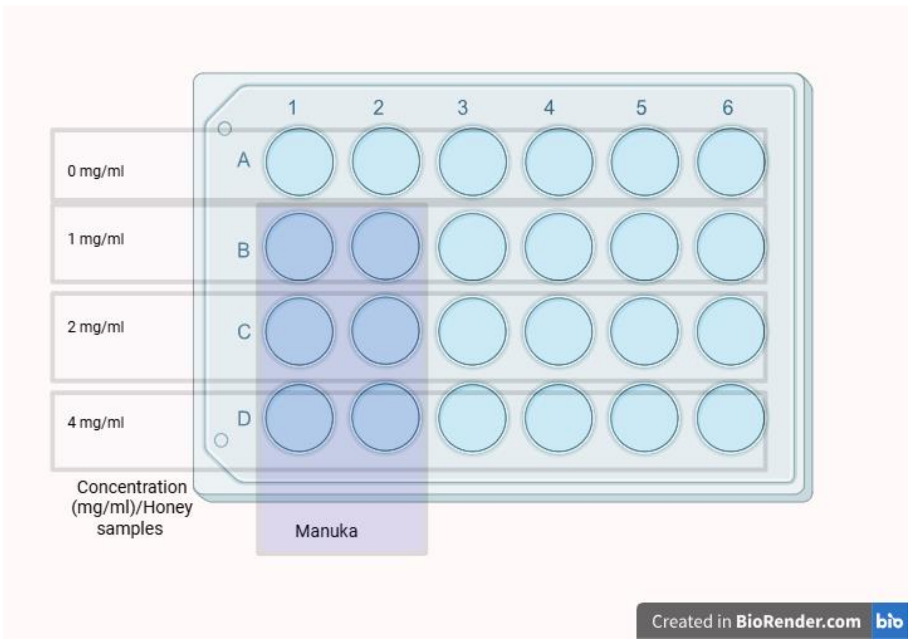

Figure 3.5: Second 24-well plate layout for wound scratch assay.

As for the nitric oxide assay, 96-well plates were pre-labeled with a plate map for observation of conditions like this:

- Standard curve: Rows A-F for columns 1-3.
- Controls: A, 4-6: cells without LPS, 7-8: cells with LPS.
- Samples for Honey types: Row A Column 10-12: Citrus, Row B, Column 4-6 : Alfalfa, Row B, Column 7-9: Avocado, Row B, Column 10-12: Manuka.

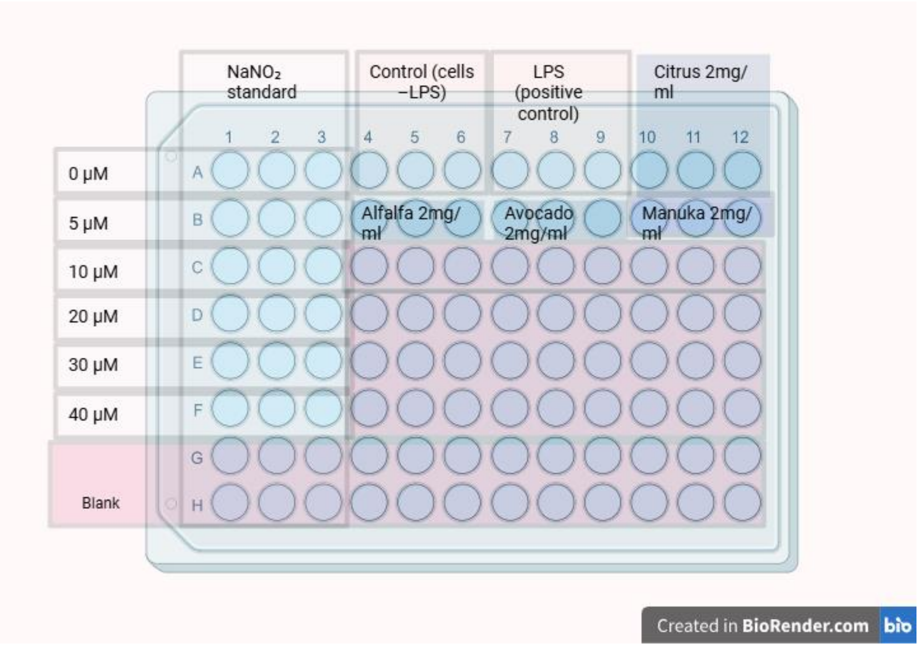

Figure 3.6: plate layout 96-well plate for nitric oxide assay.

3.6 Honey Dilution and Stock Preparation

3.6.1 Stock Solution Preparation

To facilitate reproducible in vitro delivery of honey treatments in all the assays, stock solutions of each honey type, Citrus, Alfalfa, Avocado, and Manuka, were made at a high concentration. This selection of concentration was dictated by the literature by (Chaudhary A. , Bag, Barui , Banerjee, & Chatterjee, 2015), precedent and preliminary solubility testing, and was determined to be fully soluble and compatible with culture media. This also minimized potential variations in experimental outcomes due to oxidation or microbial contamination

Stock Solution for MTT and Cytotoxicity Assays (HaCaT Cells)

Stock solutions for cell proliferation and viability assays were prepared in RPMI-1640 medium with 10% FBS. The solutions were kept only for MTT-based cytotoxicity and cytostatic assays.

Scratch (Wound Closure) Assays Stock Solution

To separate the effect of honey on cell migration from proliferation, a duplicate set of honey stocks was prepared in RPMI-1640 medium with only 2% FBS This low serum condition suppressed serum-inducible mitogenic responses but still supported basal cell survival. These honey stock preparations (100 mg/mL) were kept specifically for use in wound scratch assays.

Stock Solution for Nitric Oxide Assay (THP-1 Cells, Griess Method)

Stock solutions for the assays of nitric oxide production in macrophage-differentiated THP-1 cells were prepared in DMEM supplemented with 10% FBS. The selection of this formulation was made to maintain congruency with the standard culture conditions of THP-1 cells to avoid experiment variability resulting from media mismatch.

General Preparation Protocol

In each case, honey–medium mixtures were poured into sterile 50 mL Falcon tubes and slightly warmed at ~35°C for 10–15 minutes to reduce viscosity without denaturing heat-sensitive molecules (e.g., enzymes, antioxidants). Complete dissolution was facilitated by gentle swirling or inversion. Following solubilization, solutions were filtered through 0.22 µm syringe filters (polyethersulfone membranes) under sterile conditions to remove particulates and ensure sterility.

Filtered stock solutions were aliquoted into sterile tubes and labeled with honey type, date prepared, and medium composition, and stored at 4°C in the dark to prevent oxidation and photodegradation. Each stock was tested before use for precipitation, turbidity, or color change, which would be a sign of degradation or contamination. For each experiment, stocks were utilized within two weeks of their preparation to maintain bioactivity and reproducibility between assays.

3.6.2 Preparation of Honey Concentrations by Serial Dilution

The quantity of stock honey solution to be diluted into the final desired volume of medium was determined using the standard dilution formula:

$$C_1V_1 = C_2V_2$$

Where:

• C_1 = Initial concentration of the stock honey solution (100mg/ml)

• V_1 = Volume of the stock solution required

• C_2 = Final concentration desired (0, 2, 4, 8, 16 mg/mL)

• V_2 = Final total volume of the diluted solution

All working dilutions were prepared fresh immediately before their application to cell cultures and used within a short time window (typically under 1 hour) to prevent any degradation of honey's active molecules.

This standardized dilution protocol ensured experimental reliability, facilitated inter-experimental comparisons, and supported the accurate determination of cellular responses to the different types and concentrations of honey under investigation.

MTT Cytotoxicity and Cytostatic Assays Concentrations

For proliferation and viability assays, in RPMI-1640 medium containing 10% FBS. The honey stock solutions were serially diluted to achieve the following final working concentrations:

- 0 mg/mL (untreated control)
- 2 mg/mL
- 4 mg/mL
- 8 mg/mL
- 16 mg/mL

Scratch (Wound Closure) Assay (HaCaT cells)

To minimize serum-induced proliferation and isolate the impact of honey on migration, in RPMI-1640 medium containing 2% FBS for scratch closure experiments. Honey was applied in three final concentrations (besides the control):

- 0 mg/mL (untreated control)
- 1 mg/mL
- 2 mg/mL
- 4 mg/mL

This narrow range was chosen from pilot studies and literature reports to reflect sub-cytotoxic levels that would promote migratory activity without confounding effects of serum-induced proliferation.

Nitric Oxide Production Assay (THP-1 cells)

THP-1 monocytic cells differentiated into macrophages were grown in DMEM containing 10% FBS. In the evaluation of anti-inflammatory activity, cells were treated with honey following stimulation with LPS. One concentration of 2 mg/mL was selected for all nitric oxide (NO) assays, guided by preliminary screening and published reports. This concentration was sufficient to evaluate biological activity while minimizing the potential for cytotoxicity to differentiated macrophages.

3.7 In Vitro Assays

3.7.1 MTT Assay

Using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), cell viability and metabolic activity after honey treatment were monitored. The test is based on the reduction of MTT by mitochondrial succinate dehydrogenase in viable cells to form insoluble, colored formazan crystals, which can be dissolved and measured

spectrophotometrically. following the procedure outlined (Riss, Moravec, Niles, & Duellman, 2016).

3.7.1.1 MTT Reagent Preparation

MTT reagent was prepared as a stock concentrated solution with 5 mg/mL by dissolving 0.025 g of MTT powder in 5 mL serum-free RPMI-1640 medium. Briefly vortex the solution and protect it from light with an aluminum foil cover to avoid degradation. The working solution was prepared freshly (0.5 mg/mL) by diluting the stock 1 mL to a volume of 9 mL in serum-free medium under aseptic conditions, without contamination.

3.7.1.2 Experimental Procedure for Cytotoxicity Assay

HaCaT cells were seeded in 96-well plates (20×10^3 cells/100 µL) and incubated for 24 hr to allow the cells to adhere and grow.

Following this, the treatment medium was aspirated gently from all wells after 24 hr of incubation. The cells were washed with PBS (pH 7.4) three times to remove the remaining chemicals of the treatment, but without disorganizing the monolayer.

Dilutions of different honey types of the selected concentrations 0, 2, 4, 8, and 16 (mg/mL), in a RPMI-1640 complete growth medium, were added to the wells. After 24 hrs incubation period, the culture media were carefully removed to remove excess honey, and the cells were washed 3 times with sterile phosphate-buffered saline (PBS) to avoid any remaining traces, which could interfere with reagent penetration or absorbance readings.

Subsequently, to each well, 100 µL of a 0.5 mg/mL MTT dilution was added. All plates were then incubated at 37°C for 4 h in a 5% CO_2 cell culture incubator in the dark with foil covering. Live MTT - metabolizing cells reduced the MTT into dark purple formazan crystals during this period.

The MTT solution was then gently aspirated, and 100 µL of solubilization solution (90% isopropanol, 10% formic acid) was added to dissolve the crystals. The plate was then shaken softly on a shaker for 30 minutes at room temperature, protected from light, to attain complete solubilization and homogeneous absorbance.

3.7.1.3 Experimental Procedure for cytostatic assay

The cytostatic test is adapted as a long-term antiproliferative assessment of honey against the Human Keratinocyte HaCaT cell line by monitoring the difference in metabolic activity after prolonged incubation time.

The HaCaT cells were seeded into sterile 96-well plates (5×10^3 cells/100 µL) with complete RPMI-1640 culture medium.

Following this, the treatment medium was aspirated gently from all wells after 24 hr of incubation. The cells were washed with PBS (pH 7.4) three times to remove the remaining chemicals of the treatment, but without disorganizing the monolayer.

100 µL of different concentrations of honey 0, 2, 4, 8, and 16(mg/mL), which had been prepared afresh and sterile-filtered before use as per Section 3.6 (Honey Dilution), was added. All treatments of various concentrations were tested in technical triplicate and repeated at least 3 independent biological experiments for reproducibility.

Cells were incubated for 72 hrs following treatment to allow detection of potential cytostatic effects. Following incubation, the culture media were carefully removed to remove excess honey, and the cells were washed 3 times with sterile phosphate-buffered saline (PBS) to avoid any remaining traces, which could interfere with reagent penetration or absorbance readings.

After that, as described in the cytotoxicity assay, 100 µL of MTT solution (0.5 mg/mL in serum-free RPMI-1640) was added to wells,

Followed by incubation at 37°C for 4 hours in the dark. Metabolically active cells metabolized MTT to formazan crystals during this period. After incubation, the MTT solution was aspirated, and 100 µL of solubilization (90% isopropanol, 10% formic acid) solution was added to dissolve the crystals. Plates were shaken gently for 30 minutes at room temperature in the dark to ensure complete dissolution and homogenization.

3.7.1.4 Absorbance Measurement

Absorbance was read at 570 nm on a microplate reader (ELx800, BioTek Instruments), with comparison of the absorbance readings to cell metabolic activity. The absorbance in the treated cells was lower than that of the controls (untreated) and provided an indicator of cell growth inhibition after exposure to honey. Cells were then used for the MTT assay.

3.7.1.5 Cell Viability Calculation

Cell viability was calculated as a percentage of the mean absorbance of untreated control wells that were set at 100%. The following formula was used:

Cell Viability (%) = (Absorbance of Treated Sample / Absorbance of Untreated Control) × 100

Data are representative of three independent experiments with triplicate wells for each condition and are shown as the mean ± SD. The readings of absorbance were presented as a percentage relative to the no-treatment control group, with a value for the percent viability or growth inhibition. Data analysis was as described in Section 3.8 (Statistical Analysis).

3.7.2 Wound Healing (Scratch) Assay

The wound healing assay is an in vitro model that quantifies collective keratinocyte migration and re-epithelialization across a defined acellular gap. By introducing a linear "wound" into a confluent HaCaT monolayer mechanically and following closure over time,

the assay dissociates migratory behaviour from proliferation since it is performed under low-serum conditions and provides a direct, image-based readout of wound closure rate. In this case, the scratch assay was used to compare the migratory response of HaCaT cells upon treatment with the different preparations of honey and with the control. The wounds were imaged at 0, 24, and 48 hours and analyzed (three readings per scratch). The method is a standard practice (Liang, Park, & Guan, 2007).

3.7.2.1 Experimental Procedure Wound Healing (Scratch) Assay

The HaCaT cells 4 × 10^5 per well were seeded in a 24-well plate**,** after a complete monolayer with well-attached cells was achieved. Upon reaching optimal confluence, cells were removed using a sterile 200 microliter pipette tip to create a uniform, linear "scratch" in the cell sheet in each well. Wells were scratched individually to prevent a time lapse between scratching and washing.

Detached and non-adherent cells, along with cell debris caused by the scratch, were removed by gently washing the wells twice in 2 mL of sterile phosphate-buffered saline (PBS). This was done to ensure that only tightly attached healthy cells remained, and that the wound area would be well demarcated for imaging and measurement.

Each well was filled with 2 mL of different concentrations of tested honey types 1,2,4 (mg/ml). The concentration range was determined by cell viability results of the MTT cytotoxicity and cytostatic assays to fall within the non-cytotoxic window and to reach the right proliferative potential.

Immediately after adding treatment solutions, we imaged every wound at 10x in a microscope, and each point was precisely captured at 0, 24, and 48 hrs of treatment. Measurements were taken in three regular locations per scratch: wound width. Averaging these figures yielded the mean well width for each plate. Physical markers were attached to guides to maintain the plate positioning unchanged during the imaging process.

3.7.2.2 Wound Closer Calculation

The wound was calculated using this formula:

Percentage Wound Closure = ((Initial Wound Area - Remaining Wound Area) / Initial Wound Area) × 100

Here, Wound $Width_0h$ stands for width at 0 hours, and Wound $Width_t$ for width at 24 or 48 hours. The larger the percentage, the greater the extent of cell migration into the wound space.

A higher percentage means an increased cell migration into the wound volume. It follows that the main closure of wounds is accomplished by HaCaT cells migrating around the edge. Treatments were tested in at least three biological replicates (three different cell passages) with three technical replicates (triplicate wells) per experiment.

In this study, we describe a scratch assay that gives qualitative explanations and quantitative analysis of the nature of cell migration. Simplicity, cost-effectiveness, and ease of checking make it preferable over more complex methods such as gel transmigration assays or sandwich analyses.

3.7.3 Nitric Oxide (NO) Inhibition Assay

Following the method described by (Bryan & Grisham, 2007). The nitric oxide (NO) inhibition assay was employed to investigate the anti-inflammatory activity of honey by assessing its capacity to inhibit NO synthesis in human THP-1 macrophages stimulated with lipopolysaccharide (LPS). NO is a reactive, short-lived signaling molecule that plays a crucial role in various physiological and pathological processes. In chronic wounds, excessive generation of NO, characteristically mediated by stimulated macrophages, may contribute to inflammation and healing failure (Zhao, et al., 2025).

The honey's ability to suppress LPS-evoked NO release is therefore a valuable indicator of its putative wound-healing and inflammation-modulating activities. This assay indirectly measures NO by determining the accumulation of nitrite (NO_2^-), a stable oxidation product of NO, in the culture supernatant. Quantification was performed using the Griess reaction, a two-step diazotization process that yields a colored azo dye, measurable by spectrophotometry

Cell Seeding and Differentiation

THP-1 monocytes were cultured under normal conditions as explained in detail in 3.5.1 and differentiated to macrophage-like adherent cells before the experiment, as explained in 3.5.4. Differentiation of macrophage-like morphology was observed visually using an inverted microscope before treatment. All manipulations were carried out aseptically in a laminar flow hood to preserve culture sterility and avoid cross-contamination.

For the assay, 50,000 cells/well were seeded into 96-well flat-bottom plates for even growth and sufficient metabolic activity during the treatment period without overconfluence. Cell suspension was gently pipetted into wells to prevent uneven distribution, and the plates were incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere to facilitate cell adherence and recovery

Treatment Conditions

Following adherence, culture medium was aspirated gently from all wells, and cells were treated with the given 2ml of the different tested honey. Treating was done in triplicate for each tested concentration. To induce the generation of NO, LPS was introduced in all wells except for unstimulated negative controls.

Positive inflammatory controls were cells treated with LPS but no honey, and served as the standard for maximum NO production. After adding treatments, plates were returned to the CO_2 incubator and stored under standard conditions for 72 hours of incubation time, during which cells were undisturbed.

Collection of Supernatants

Supernatants of cell cultures at the end of treatment time were collected carefully to quantify nitrite. Avoiding the dislodgement of the cell monolayer or introduction of cellular debris into the assay, pipette tips were positioned over the layer of cells, and gradually the supernatant was withdrawn. Fifty microliters of every supernatant sample were added to a fresh 96-well flat-bottom plate that would be used for the Griess reaction. The samples were treated with care on collection to prevent any alteration of the concentration of nitrite on storage.

Griess Reaction Procedure

The Griess reaction was carried out in two sequential steps to maximize sensitivity and consistent reaction kinetics in every well. A 100 µL of the first reagent (Reagent B, sulfanilamide in phosphoric acid) was directly pipetted into the 50 µL sample in every well. The first step forms the diazotization reaction between nitrite and sulfanilamide under acidic conditions, forming a diazonium salt. After a gentle tap of the plate to mix the contents, 100 µL of the second reagent (Reagent A, N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The second reaction pairs the diazonium salt with NED to produce a chromophoric azo compound, whose coloration is pink–purple and whose color intensity is in direct proportion to the nitrite concentration present. The two additions of reagents were carried out in reduced light to prevent photodegradation. The addition order and timing between steps were also standardized across all wells carefully to minimize variability.

Incubation for Color Development

Following the final reagent addition, plates were incubated for 10 minutes at room temperature in the dark to achieve maximum color development. Plates were not touched during the incubation time to avoid the introduction of microbubbles that can interfere with absorbance readings or uneven mixing. Maximum total reaction time was utilized to allow a compromise between maximum intensity of the color and stability of reagents because additional incubation time beyond the recommended time could lead to loss of signal or noise in the background.

Absorbance Measurement

After incubation, absorbance at 540 nm was measured using an ELISA microplate reader. Wells were checked for visible precipitation, bubbles, or optical path anomalies that could interfere with the measurement and were excluded or re-read accordingly. A sodium nitrite standard curve was prepared in parallel with the same assay reagents and culture medium to facilitate proper interpolation of nitrite concentrations in test samples. Standards ranged from 0–40 μM to capture expected treatment responses.

The Standard curve preparation

To accurately quantify the amount of nitrite in experimental samples, which is considered unknown, a sodium nitrite ($NaNO_2$) standard curve with known nitrite amounts was generated for each assay run. A concentrated stock solution of $NaNO_2$ was first prepared at a concentration of 100 mM in distilled water, mixed gently to achieve complete dissolution. This stock was used to prepare a series of working standard solutions by serial dilution in the same culture medium used for cell treatments to minimize matrix effects on color development in the Griess reaction. The final nitrite concentrations in the standard set were 0, 5, 10, 20, 30, 40, and 50 μM, spanning the range of nitrite accumulation observed in LPS-stimulated macrophage cultures.

The standards prepared were transferred in triplicate into the wells of a new 96-well plate, with each volume being equal to that used for experimental samples, to maintain equivalent assay conditions. The Griess reaction was developed on these standards in parallel to test samples, and the absorbance was read under identical conditions on the microplate reader. By plotting the absorbance values against known nitrite concentrations, a standard curve was prepared and used to identify by interpolation the nitrite content in unknown samples. The linearity of the curve was verified beforehand, and any assay with an R^2 value below 0.99 was re-run to ensure the accuracy and reliability of the quantitation procedure. These procedures are detailed in Table 3. 2 Summary of Assay Conditions

Table 3.2: provides an overview of the cell types, seeding densities, honey concentrations, incubation times, and readouts for each in vitro assay performed in this study.

Assay	Cell Type	Seeding Density	Honey Concentrations	Incubation Time	Readout
MTT (Cytotoxic)	HaCT keratinocytes	20×10^3 cells/well (96-well)	0, 2, 4, 8, 16 mg/mL	24hr treatment	Absorbance at 570nm (formazan quantification)
MTT (Cytostatic)	HaCaT keratinocytes	5×10^3 cells/well (96-well)	0, 2, 4, 8, 16 mg/mL	72hr treatment	Absorbance at 570nm (formazan quantification)
Scratch (Wound Closure)	HaCaT keratinocytes	4×10^5 cells/well (24-well, confluent monolayer)	1, 2, 4 mg/mL (2%FBS medium)	0 hr, 24 hr, 48hr imaging	ImageJ wound width analysis (% closure)
Nitric Oxide (Griess)	THP-1 macrophages	50,000 cells/well (96-well) after PMA differentiation	2 mg/mL (after LPS stimulation)	72hr post-treatment	Absorbance at 540 nm (nitrite quantification with $NaNO_2$ standard curve)

3.8 External assays

To support and augment our research, additional collaborative experiments were performed. Molecular docking analyses of selected honeys were performed by Dr. Hamada Imtara at the Faculty of Medicine, Arab American University (Jenin), and were found to be beneficial mechanistic data that supported our findings. In addition, antimicrobial activity was determined in collaboration with Dr. Basheer Abu-Farich and Dr. Mahmoud Masalha at

the Al-Qasemi Research Center, where routine microbiological tests were performed against clinically relevant pathogens.

3.8.1 Preliminary Antimicrobial Testing

Antimicrobial screening was conducted in order to experimentally determine the antibacterial activity of selected honey samples (Avocado, Citrus, Alfalfa) against a collection of clinically significant bacterial reference organisms. Through standardized broth microdilution assays with 96-well plates following the (Abu-Farich, et al., In vitro evaluation of Hyphaene thebaica honey as a multitarget therapeutic product, 2025) procedure, minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) were measured along with the and IC_{50} values from concentration–response profiles. Assays were performed under organism-compatible growth conditions and media, in technical triplicate and separate biological repeats, and with growth, sterility, and antibiotic quality-control controls to ensure assay integrity.

Bacterial strains.

The following reference strains were used: 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 Bacillus subtilis (ATCC 6633). All strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were stored, revived and maintained according to ATCC recommendations prior to testing.

Preparation of bacterial inocula.

Fresh overnight cultures were prepared on appropriate solid media from single colonies. Colonies were suspended in sterile saline and adjusted to a 0.5 McFarland standard (≈1–2 × 10^8 CFU/mL). Working inocula were prepared by diluting this suspension in the

appropriate broth to obtain a final concentration of ≈5×10^5 CFU/mL in each well of the microtiter plate.

Media and special growth requirements.

Mueller–Hinton broth (MHB) was used for routine testing of P. aeruginosa, S. aureus, E. coli, K. quasipneumoniae, and B. subtilis. For fastidious organisms, media were supplemented according to standard guidance: S. pneumoniae testing was performed in MHB supplemented with 5% lysed horse blood, and H. influenzae testing was performed in MHB/Haemophilus Test Medium (or MHB supplemented with NAD and hemin) to meet its growth requirements. Incubation conditions followed CLSI-recommended atmospheres and temperatures (35 ± 2 °C; 5% CO_2 for S. pneumoniae and H. influenzae, ambient air for others) and the incubation times described below.

Honey sample preparation.

Honey samples (Citrus, Alfalfa, Avocado) were weighed and dissolved in sterile distilled water to prepare a concentrated stock (w/w%). Stocks were vortexed until homogeneous. Stocks were filter-sterilized where feasible (0.22 µm syringe filter) Two-fold serial dilutions of each honey stock were prepared in the appropriate broth in sterile 96-well microtiter plates to cover the working range used in this study (final tested concentrations ranged from 50% to 0.1953125% w/w, prepared as serial two-fold dilutions to capture MIC/MBC and IC_{50} values reported).

MIC determination (broth microdilution).

MIC was recorded as the lowest concentration of honey that completely inhibited visible growth (no turbidity) compared with the growth control.

Two-fold serial dilutions of each honey were prepared in the appropriate broth across the plate (final well volumes 100 µL).

100 µL of bacterial working inoculum (≈1 × 10^6 CFU/mL) were added to each well to give a final inoculum of ≈5 × 10^5 CFU/mL and the target honey concentration. Controls included on each plate: growth control (inoculum + broth, no honey), sterility control (broth only), and a vehicle control (if applicable). An antibiotic control (known active antibiotic) was included periodically to verify assay performance.

Plates incubated at 35 ± 2 °C for 18–24 hours, except for S. pneumoniae and H. influenzae, which were incubated under 5% CO_2 for 18–24 hours.

MBC determination.

Minimum bactericidal concentrations (MBCs) were determined from wells showing no visible growth in the MIC assay, MBC was defined as the lowest concentration of honey resulting in ≥99.9% reduction in CFU (no colony growth on the plate or ≥3-log reduction compared with the starting inoculum).

From wells at and above the MIC, 10 µL aliquots were aseptically plated onto the appropriate non-selective agar (e.g., Mueller–Hinton agar; chocolate agar for H. influenzae / blood agar for S. pneumoniae) and incubated under organism-specific conditions. Plates were inspected after 18–48 hours, depending on the organism's growth rate.

IC_{50} calculation.

IC_{50} values were estimated from concentration-response data. Experiments for IC_{50} determination were performed in triplicate, and dose-response curves were fitted using nonlinear regression (four-parameter logistic model) in statistical software to obtain IC_{50} values and 95% confidence intervals.

Replicates and quality control.

All MIC/MBC determinations were performed in technical triplicate and at least two independent biological repeats on separate days. ATCC quality-control strains and previously reported reference MIC values were used to verify assay validity, and any plate failing QC criteria was repeated.

3.8.2 Molecular Docking (in silico)

Docking of molecules was performed to predict binding modes and relative binding affinities of six phenolic compounds from avocado honey towards four protein targets (glutathione reductase 3GRS, dihydrofolate reductase 4M6J, 5-lipoxygenase 1N8Q, and EGFR 1XKK). Docking calculations and map preparation were performed with AutoDock4/AutoDockTools (using the Lamarckian Genetic Algorithm, LGA, and AutoGrid) to allow for flexible-ligand docking and uniform grid map preparation following the method (Morris, et al., 2009).

Preparation of protein.

Crystal structures were retrieved from the Protein Data Bank and processed by their removal of crystallographic waters and unbound ligands, addition of all polar hydrogens, addition of Kollman united-atom charges, and merging of non-polar hydrogens as needed. Proton states were checked at physiological pH, and any missing side chains were modelled before saving the receptor in PDBQT format (AutoDockTools).

Ligand preparation.

The 2D structures of the six phenolics were converted to 3D and energy-minimized. Gasteiger charges were assigned, and rotatable bonds were defined for each ligand. Ligands were saved in PDBQT format with torsional degrees of freedom enabled for flexible docking.

Grid definition and configuration files.

For each target, the grid box was placed in the center of the known active site (co-crystallized ligand or catalytic residues). AutoGrid was used to generate grid maps for each type of atom. To maintain runs reproducible and comparable among each other, all grid parameters (centre coordinates, number of grid points, and spacing) and docking parameters were retained in config files and imposed unrevised over compounds. Grid spacing was 0.375 Å (AutoDock default), and box size was chosen to fully enclose the active site plus some buffer space for ligand flexibility (usual box sizes ~40 × 40 × 40 points; actual values recorded in the configuration files for each protein).

Docking parameters.

Docking utilized the Lamarckian Genetic Algorithm (LGA) implemented in AutoDock4. Search conditions were kept the same for all ligands: number of GA runs = 100, population size = 150, max number of energy evaluations = 2,500,000, max number of generations = 27,000, and clustering RMSD tolerance = 2.0 Å. Best-scoring poses were selected by predicted binding energy (most negative ΔG). Employing the same GA and grid parameters for all ligands guaranteed that variations in binding energy are a consequence of ligand–target complementarity, not variable sampling.

3.9 Statistical Analysis

Data from the three assays, MTT cell viability and proliferation, scratch wound healing, and Griess nitric oxide, were first analyzed in Microsoft Excel to calculate mean values and standard deviations (SD), and to perform initial pairwise comparisons between treatment groups and the control. Graphical representations were initially created with Excel for a preliminary overview. For publication-quality figures and further visualization, the data were imported into R statistical software (version X.X, R Foundation for Statistical Computing, Vienna, Austria) using packages such as ggplot2 for graphing.

3.9.1 Preprocessing and Normalization of Raw Data

MTT Cytotoxic and Cytostatic Assays

The raw absorbance values were read from each well and recorded at a wavelength of 570 nm. Before proceeding with further calculations, the absorbance values from blank wells containing only culture medium were subtracted. The adjusted result is reported as corrected absorbance, eliminating background signals from the plastic itself as well as any residual breakdown products of phenol red. These values were standardized to the untreated control and expressed as 100% cell viability. Cell viability for each honey treatment and concentration was determined as a percentage relative to the control group. Cytostatic and cytotoxic endpoints were both derived from these standardized figures. Cytotoxicity refers to direct reductions in viable cell number, while cytostaticity refers to inhibition of proliferation without marked cytotoxicity

Scratch Wound Closure Assay

Scratch wounds were quantified by capturing the image of three points of wound width during 0,24,48 hrs. The width or area of the scratch was measured at 0 hours and then reassessed at 24 h and 48 h after drug treatment. Percent closure was calculated using the formula:

Percent closure (%) = ((Initial scratch area – Remaining scratch area) / Initial scratch area) × 100.

These percentages were averaged over the repeats per condition. The data were normalized against the untreated control, which was used as a standard for wound healing and spontaneous migration.

Nitric Oxide (Griess) Assay

Absorbance readings at 540 nm were taken and converted into nitrite concentrations (μM) based on a sodium nitrite ($NaNO_2$) standard curve (0-100 μM). These concentrations were also expressed as a percentage of the basal control (unstimulated cells being 100%). In

addition, honey-treated cells were compared with the positive control (LPS-stimulated cells), which indicated maximal levels of inflammatory activation. This allowed both honey treatment effects to be examined in terms of induction above baseline and suppression relative to LPS-induced groups. The equations used:

1. Conversion from absorbance to nitrite concentration using the standard curve:

$$[\text{Nitrite}]\ (\mu M) = (A_{540} - b) / m$$

Where A_{540} = absorbance at 540 nm, m = slope of standard curve, b = intercept.

2. Normalization relative to the untreated control (set as 100%):

$$\%\ \text{NO Production} = ([\text{Nitrite}]_{(\text{sample})} / [\text{Nitrite}]_{(\text{control})}) \times 100$$

Antimicrobial (MIC/MBC and IC_{50})

For antimicrobial assays against the exterior, triplicate MIC, MBC, and IC_{50} measurements were initially averaged, and SD values were calculated using Excel. MIC and MBC (w/w %) values were provided. Independent two-tailed t-tests were performed in R for comparisons between inter-honey type and inter-bacterial strains. Lower MIC, MBC, or IC_{50} values indicated higher antibacterial activity. Graphs were produced using ggplot2 for graphical comparison of bacterial susceptibility patterns between honey samples.

Molecular Docking

AutoDock Vina-calculated docking scores Docking scores (binding energies, Be [$kcal \cdot mol^{-1}$]) and estimated inhibitory concentrations (Ic [µM]) were calculated using AutoDock Vina. For each of the target–compound pairs, there were several docking runs (n = 100), and the lowest-energy pose was selected for analysis. Descriptive statistics were calculated on replicate runs. Estimated Ic values were calculated from Be ,For ΔG in $kcal \cdot mol^{-1}$:

$$Ki(M)=\exp(\Delta G\times1000/(R\times T)),\ R=1.987\ cal\backslash\backslash\text{-}pmol^{-1}\backslash\backslash\text{-}K^{-1}, T=298.15K$$

Values were divided by 10^6 to report Ic in µM. Docking poses were validated by superposition with crystallographic ligands (where available) and inspected in PyMOL and

2D interaction plots (Discovery Studio / LigPlot) to identify hydrogen bonds, hydrophobic contacts, π–π/π–alkyl contacts, and stabilizing contacts. Before screening, the docking protocol was validated by re-docking co-crystallized ligands (when available) and verification of RMSD $\leqslant$ 2.0 Å between predicted and crystallographic poses

3.9.2 Statistical Analyses

MTT Assay

Cell viability data after 24 or 72 hours were expressed as the mean ± SD. The honey-treated groups were compared to the untreated control group at the same concentration or time by simple pairwise comparisons. When appropriate, a similar comparison was also made among different concentrations within its own type of honey. These comparisons were done using independent two-tailed t-tests. A significance level of $p < 0.05$ was adopted.

Scratch Wound-Healing Assay

Wound closure values were expressed as mean ± SD. Pairwise comparisons were made at each time point (24 h and 48 h) to evaluate any differences between the honey-treated groups and the control group. At the same concentration or time-point, comparisons between two types of different honeys were also conducted using independent t-tests. A significance level of $p < 0.05$ was adopted.

Nitric Oxide (Griess) Assay

Nitrite concentrations were calculated from the sodium nitrite standard curve and expressed as mean ± SD. Comparisons of NO production were made between honey-treated groups and the control (unstimulated cells) as well as the LPS-stimulated positive control. Statistical comparisons between groups were performed using independent t-tests. A significance level of $p < 0.05$ was adopted.

Antimicrobial (MIC/MBC and IC_{50})

MIC, MBC, and IC_{50} values were given as mean of triplicate determinations. Independent two-tailed t-tests were employed for comparison of antibacterial activity in

honey-treated groups with the control, and among different types of honey tested for antibacterial activity against a single microorganism. Lower MIC, MBC, and IC_{50} values were considered to represent higher antimicrobial activity. A cut-off value of $p < 0.05$ was employed.

Molecular Docking

Binding energy (Be, $kcal \cdot mol^{-1}$) and inhibitory concentration (Ic, μM) values obtained by docking simulation were given as the mean. Docking affinities between two sets of compounds for a single target protein were compared using independent two-tailed t-tests for statistical analysis. All those with lower Be and Ic values were considered to have greater predicted binding affinity. In addition, in-silico docking correlations with experimentally measured antimicrobial values (MIC or IC_{50}) were interrogated with independent t-tests wherever feasible. A significance level $p < 0.05$ was employed.

4 Chapter Four: Results

This chapter presents experimental results from in vitro assays examining the effects of three honey types, citrus, alfalfa, and avocado, on HaCaT human keratinocytes. In particular, findings of the MTT assay (cell viability and proliferation), the scratch wound-closure assay (cell migration and wound healing), and the Griess nitric-oxide assay (nitric-oxide–mediated inflammatory response) are reported. All findings are plotted over the concentration range examined as figures and tables with relevant summary statistics.

Additionally, the antibacterial efficacy of the honey samples was ascertained through MIC, MBC, and IC_{50} estimates against clinically relevant bacterial strains, and their results are listed for relative comparison between honeys. Molecular docking simulations for Avocado honey polyphenolic compounds were carried out to project future potential binding with prominent protein targets, providing computational predictions of noted bioactivity.

4.1 General reporting conventions

Unless otherwise indicated, all values are expressed as mean ± SD of n =3 which are independent biological replicates. Values exceeding 100% indicate increased cell viability or proliferation relative to the untreated control. Experimental and analytical methods are described in Chapter 3 (Materials and Methods). Statistical comparisons among treatment groups were made using the unpaired Student's t-test, and $p < 0.05$ was considered statistically significant. Error bars in figures represent SD. Figures 4.1–4.17 and Tables 4.1–4.10 present the key data and statistical comparisons. Their interpretation in relation to the study hypothesis and literature is postponed to Chapter 5.

4.2 Cell viability & proliferation — MTT assay

MTT assay was employed to assess the effect of Citrus, Alfalfa, Avocado, and Manuka honey on HaCaT keratinocyte viability and proliferation, with cytotoxicity and cytostasis measurement. The cells were treated with honey concentrations of 0, 2, 4, 8, and 16 mg/mL, and viability was tested after 24 hours for cytotoxicity and 72 hours for cytostatic. The percentages, with respect to the untreated control, are reported in Table 4.1-4.4 and illustrated in Figure 4.1-4.4, in which the influence of each kind of honey on keratinocyte proliferation and survival is reported.

4.2.1 Citrus Honey

Citrus honey effects on HaCaT keratinocyte viability, as analyzed by the MTT assay. Values exceeding 100% indicate increased cell proliferation relative to the untreated control. are shown in Figure 4.1. Increasing concentrations of Citrus honey resulted in reduced cytostatic activity, with viability decreasing from 100% in the controls to 53.6% at 16 mg/mL. The lower cytotoxic effects were noted, with viability decreasing from 100% (control) to 87.5% at the maximum used concentration. Reduction in cytostatic values was greater than the corresponding variations in cytotoxicity at every concentration. Table 4.1 presents the results.

Table 4.1: Cell viability of cytotoxic and cytostatic assay for citrus honey.

Concentration (mg/mL) of Citrus Honey	**Cell Viability (% of Control) – Cytotoxic± SD**	**Cell Viability (% of Control) – Cytostatic± SD**
0	100 ± 10	100 ± 2
2	75 ± 5	90 ± 2
4	55 ± 7	90 ± 2
8	55.8 ± 8	92.25± 2
16	53.6 ± 8	87.5± 2

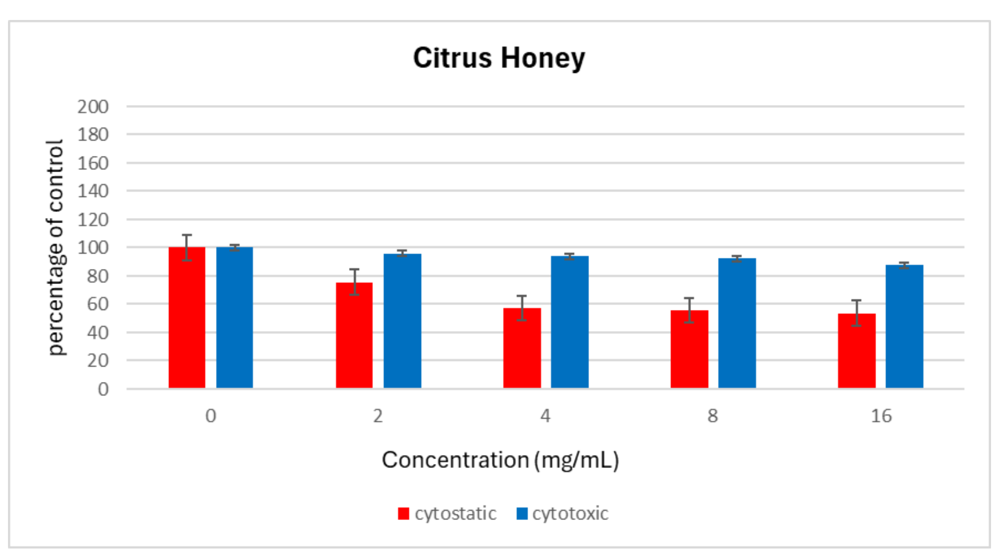

Figure 4.1: Citrus honey cytostatic (red) and cytotoxic (blue) activity against HaCaT keratinocytes. Data show percentage of untreated control (mean ± SD, n = 3). *$p < 0.05$ vs control (unpaired Student's t-test).

4.2.2 Alfalfa Honey

Alfalfa honey is observed to exert cytostatic and cytotoxic effects in Figure 4.2. At lower concentrations (2–8 mg/mL), cytostatic activity was above control and was greatest at 2 mg/mL (187%), then it dropped a little bit at 4 mg/mL (160%). Cytotoxic effects remained low up to 8 mg/mL, but viability fell to 96.5% at 16 mg/mL. Alfalfa honey generally demonstrated a biphasic cytostatic response, with stimulation occurring at lower concentrations and inhibition at higher concentrations, whereas cytotoxicity only increased with the highest concentration employed. Table 4.2 presents the results.

Table 4.2: Cell viability of cytotoxic and cytostatic assay for Alfalfa honey.

Concentration (mg/mL) of Alfalfa Honey	Cell Viability (% of Control) – Cytotoxic± SD	Cell Viability (% of Control) – Cytostatic± SD
0	100 ± 2	100 ± 20
2	100 ± 2	185 ± 15
4	95 ± 2	160 ± 20
8	93 ± 5	105 ± 20
16	96 ± 2	80 ± 20

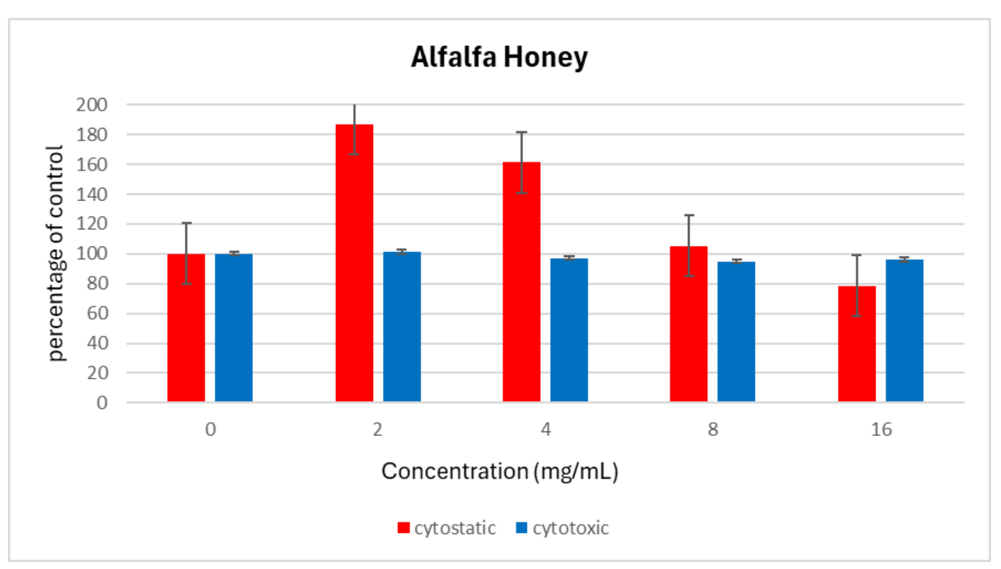

Figure 4.2: Cytostatic (red) and cytotoxic (blue) activity of Alfalfa honey in HaCaT keratinocytes. Data are expressed as a percentage of untreated control (mean ± SD, n = 3). *p < 0.05 vs control (unpaired Student's t-test).

4.2.3 Avocado Honey

Avocado honey is observed to exert cytostatic and cytotoxic effects in Figure 4.3. At lower concentrations (2–8 mg/mL), cytostatic activity was above control and was greatest at 2 mg/mL (167%). Cytotoxic effects remained low up to 8 mg/mL, but viability fell to 76.5% at 16 mg/mL. Avocado honey generally demonstrated a biphasic cytostatic response, with stimulation occurring at lower concentrations and inhibition at higher concentrations, whereas cytotoxicity only increased with the highest concentration employed.

Table 4.3: Cell viability of cytotoxic and cytostatic assay for Avocado honey.

Concentration (mg/mL) of Avocado Honey	**Cell Viability (% of Control) – Cytotoxic± SD**	**Cell Viability (% of Control) – Cytostatic± SD**
0	100 ± 20	100 ± 2
2	97.85 ± 5	167 ± 15
4	94.3 ± 5	135 ± 20

8	90.7 ± 5	128.6 ± 20
16	76.5 ± 5	76.5 ± 15

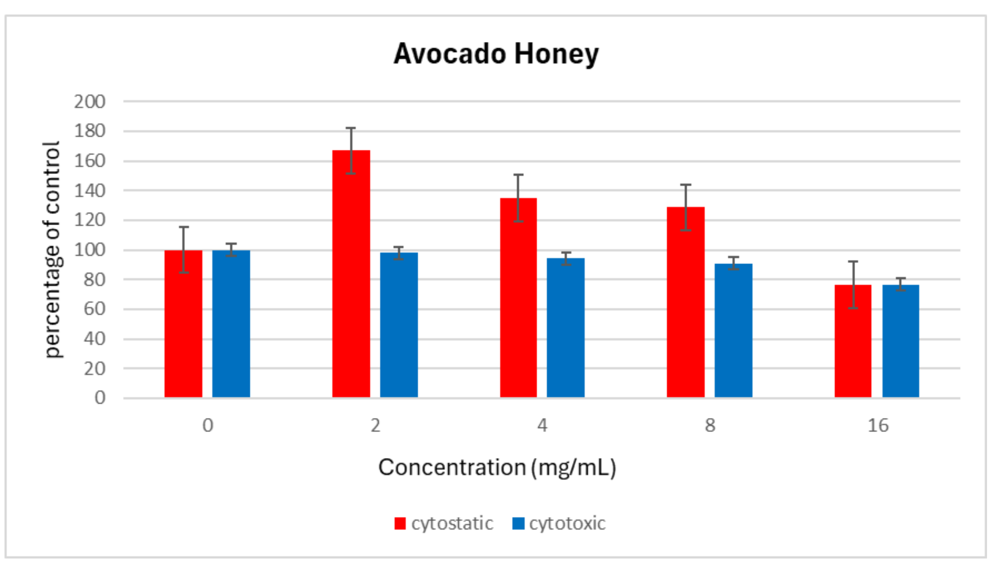

Figure 4.3: Cytostatic (red) and cytotoxic (blue) activity of avocado honey in HaCaT keratinocytes. Data are expressed as a percentage of untreated control (mean ± SD, n = 3). *p < 0.05 vs control (unpaired Student's t-test).

4.2.4 Manuka Honey

Manuka honey is observed to exert cytostatic and cytotoxic effects in Figure 4.4. At lower concentrations (2–8 mg/mL), cytostatic activity was above control and was greatest at 2 mg/mL (159%). Cytotoxic effects remained low up to 8 mg/mL, but viability fell to 76.5% at 16 mg/mL. Manuka honey generally demonstrated a biphasic cytostatic response, with stimulation occurring at lower concentrations and inhibition at higher concentrations, whereas cytotoxicity only increased with the highest concentration employed.

Table 4.4: Cell viability of cytotoxic and cytostatic assay for Manuka honey.

Concentration (mg/mL) of Manuka Honey	Cell Viability (% of Control) – Cytotoxic± SD	Cell Viability (% of Control) – Cytostatic± SD

0	100 ± 20	100 ± 2
2	90.5 ± 2	159 ± 20
4	76.4 ± 5	104.5 ± 20
8	86.7 ± 2	63.3 ± 20
16	74.8 ± 2	56 ± 20

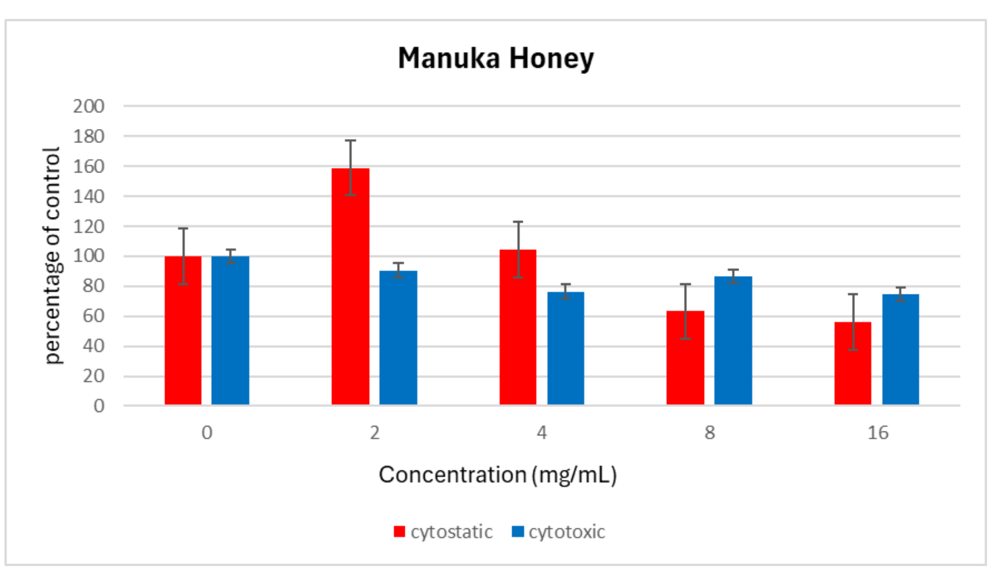

Figure 4.4: Cytostatic (red) and cytotoxic (blue) activity of manuka honey in HaCaT keratinocytes. Data are expressed as a percentage of untreated control (mean ± SD, n = 3). *$p < 0.05$ vs control (unpaired Student's t-test).

4.2.5 Summary of Cell Viability and Proliferation Findings

The MTT assay revealed that all honeys tested—Citrus, Alfalfa, Avocado, and Manuka—had measurable effects on HaCaT keratinocyte viability, including cytostatic and cytotoxic effects of varying concentration dependence. These findings demonstrate distinct concentration-dependent effects of each honey on keratinocyte growth and survival.

- Citrus Honey:

Treatment with Citrus honey revealed a steady reduction in cytostatic activity with increasing concentration. Cytostatic levels fell from 100% in untreated control cells to 87.5% at the concentration of 16 mg/mL. Cytotoxicity was relatively low at all test concentrations. In general, Citrus honey functioned primarily to inhibit cell growth without having major cytotoxic effects.

- Alfalfa Honey:

Alfalfa honey exhibited a biphasic cytostatic response. At lower concentrations, cytostatic activity was elevated above control cells, showing proliferation stimulation of keratinocytes at these levels. The cytostatic activity declined with increasing concentrations. Cytotoxicity was minimal up to 8 mg/mL but dropped to 78.5% at 16 mg/mL. These results indicate that Alfalfa honey is capable of increasing cell growth at lower dosages and mildly inhibiting it at higher dosages.

- Avocado Honey:

Cytostatic effect was enhanced in low concentrations, up to 167% at 2 mg/mL, but progressively reduced with increasing concentrations. Cytotoxicity remained low at low to intermediate concentrations but was evident at the highest concentration (16 mg/mL), with cell viability reduced to 76.5%. These results show that Avocado honey is potentially proliferative at low doses and weakly inhibitory and cytotoxic at higher doses.

- Manuka Honey:

Manuka honey exhibited a biphasic response to cytostatic activity, with optimal stimulation occurring at 2 mg/mL (159%). Cytostatic activity was decreased at elevated concentrations, indicating proliferation inhibition. Cytotoxicity was more pronounced with growing concentration, with viability decreasing to 56% with the

highest concentration tested (16 mg/mL). Manuka honey tended to exhibit a larger inhibitory effect on cell viability at higher concentrations than the other honeys tested, although low concentrations still caused proliferation.

4.3 Wound Scratch Assay

The wound scratch assay was used to test the effect of different honey samples on HaCaT keratinocyte migration. Treatments were applied at concentrations of 1, 2, and 4 mg/mL of honey. Wound healing was monitored at 24 h and 48 h post-treatment. Results are represented as a percentage of initial wound area covered, and are illustrated in Figures 4.5-4.13. Figure 4.5 shows media control containing 0 mg/mL of honey and 2% serum, which will be compared with all samples, showing the difference in the scratch area over the 24-48 hrs.

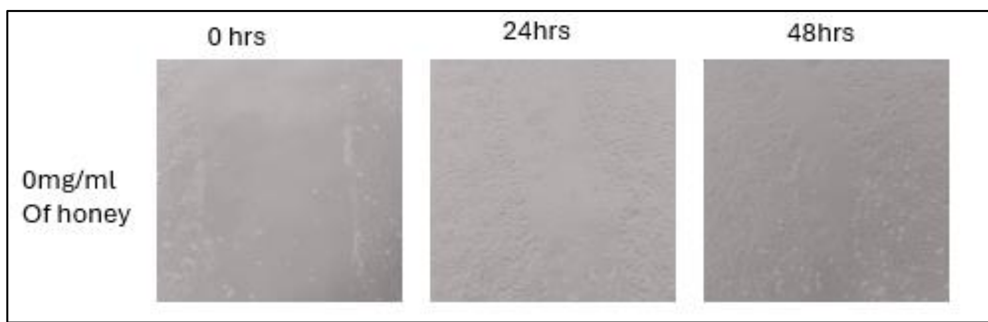

Figure 4.5: 0 mg/mL control, media effect in wound scratch area 0,24, 48 hrs. Wound area was measured as a percentage of the initial scratch at 0 h. Data represent baseline migration without honey treatment (mean ± SD, n = 3).

4.3.1 Citrus Honey

Citrus honey promoted HaCaT migration at all concentrations, as shown in Figure 4.7. After 24 h, the rate of wound closure was fixed at 100% at 1 mg/mL and 4 mg/mL. After 48 h, the rate of wound closure was greater, ranging from 113% to 127%, with maximal closure at 4 mg/mL. This indicates that citrus honey stimulates progressive cell migration in the long term, and the effects are more evident at the later time point. Figure 4.6 shows representative scratch assay images at 0, 24, and 48 h. The results presented in Table 4.5 indicate that citrus honey has a moderate, long-term stimulatory effect on wound healing.

Table 4.5: Effect of citrus honey on HaCaT keratinocyte migration in the scratch wound-healing assay at 24 and 48 hours. Values are expressed as % of control (mean ± SD, n = 3).

Citrus Honey concentration (mg/mL)	24 hrs (% migratory cells) ± SD	48 hrs (% migratory cells) ± SD
Control	100 ± 5	102 ± 6
1	102 ± 6	115 ± 7
2	110 ± 5	128 ± 8
4	104 ± 6	121 ± 7

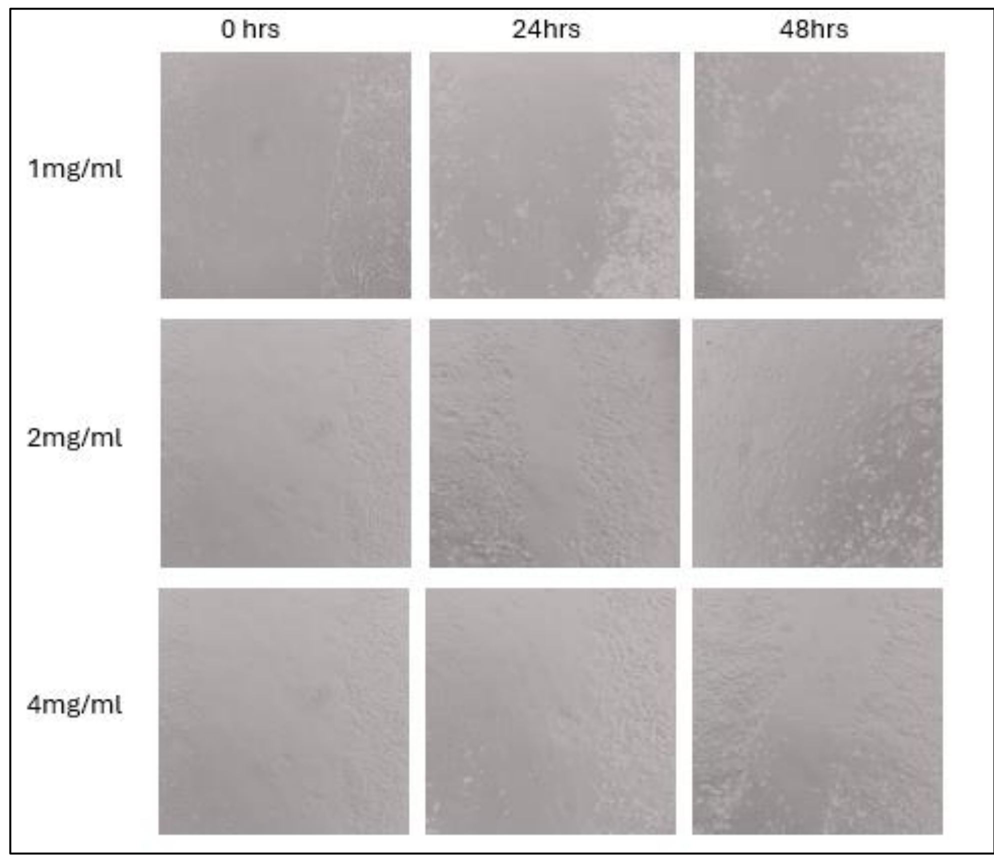

Figure 4.6: Representative micrographs from the scratch wound-healing assay of HaCaT keratinocytes treated with citrus honey at different concentrations (1, 2, and 4 mg/mL). Images were taken at 0, 24, and 48 hours after scratch creation.

Figure 4.6. Representative micrographs from the scratch wound-healing assay of HaCaT keratinocytes treated with citrus honey at different concentrations (1, 2, and 4 mg/mL). Images were taken at 0, 24, and 48 hours after scratch creation. Quantification of percentage wound closure is shown in Figure 4.7

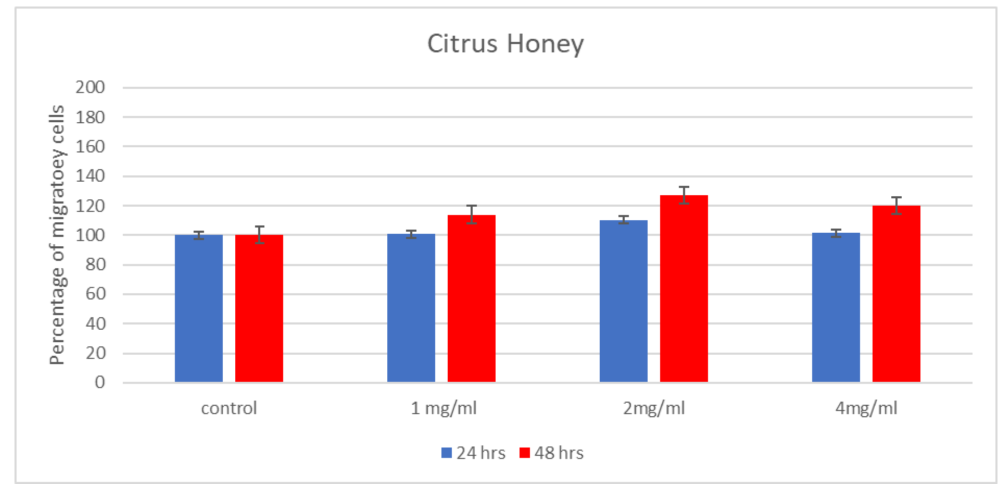

Figure 4.7: Quantification of wound closure as a percentage of migratory HaCaT cells during 24 and 48 hrs, for Citrus honey. Data are expressed as a percentage of untreated control (mean ± SD, n = 3). *p < 0.05 vs control (unpaired Student's t-test).

4.3.2 Alfalfa Honey

Alfalfa honey had a greater wound closure effect compared to citrus honey, as demonstrated in Figure 4.9. The results presented in Table 4.6 indicate that at 24 h, closure was 115% at 1 mg and dropped as the concentration increased; the peak closure occurred at 1 mg/mL. At 48 h, it started at 145%, indicating that there is a sustained stimulatory effect on migration over time.

As predicted, maximal migration (4 mg/mL) was less active than the lower concentrations at both periods (24 -48), and the inference is that optimal migration is perhaps accomplished at intermediate honey concentrations. Overall, alfalfa honey promoted initial cell migration and helped preserve wound closure by 48 hr. Figure 4.8 shows scratch assay images at 0, 24, and 48 h.

Table 4.6: Effect of Alfalfa honey on HaCaT keratinocyte migration in the scratch wound-healing assay at 24 and 48 hours. Values are expressed as % of control (mean ± SD, n = 3).

Alfalfa Honey concentration (mg/mL)	24 hrs (% migratory cells) ± SD	48 hrs (% migratory cells) ± SD
Control	105 ± 10	100 ± 8
1	115 ± 12	145 ± 10
2	95 ± 10	105 ± 10
4	65 ± 8	95 ± 9

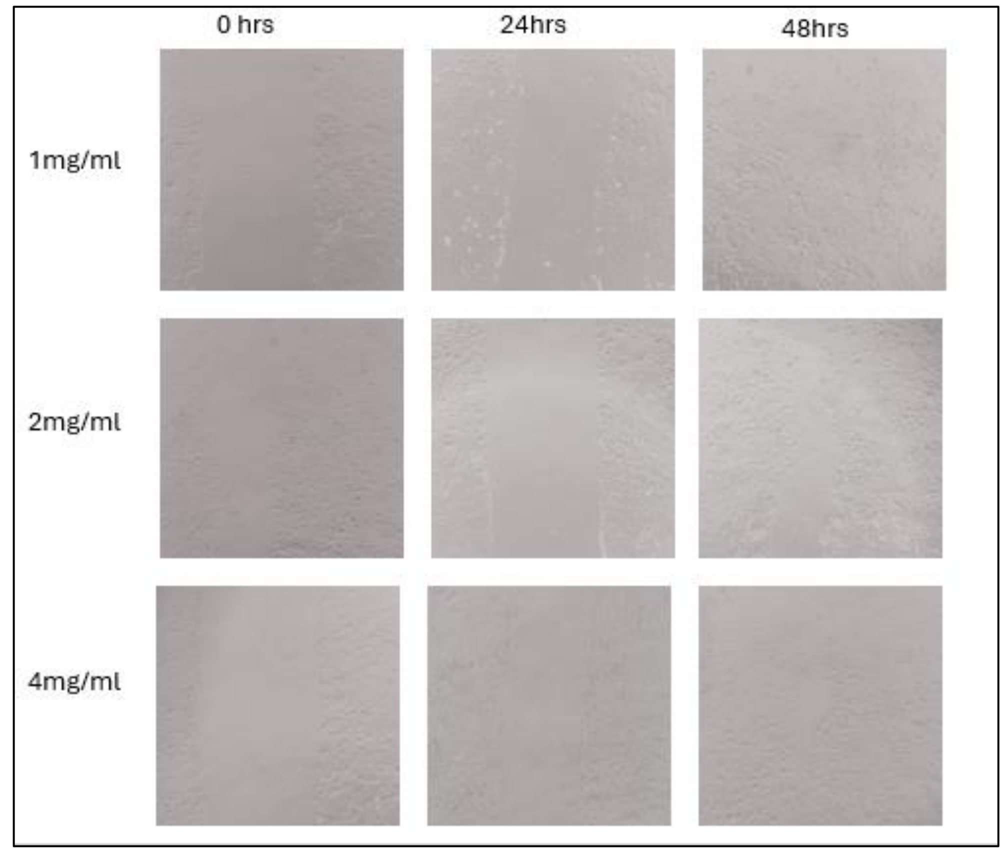

Figure 4.8: Representative micrographs from the scratch wound-healing assay of HaCaT keratinocytes treated with Alfalfa. Images were taken at 0, 24, and 48 hours after scratch creation.

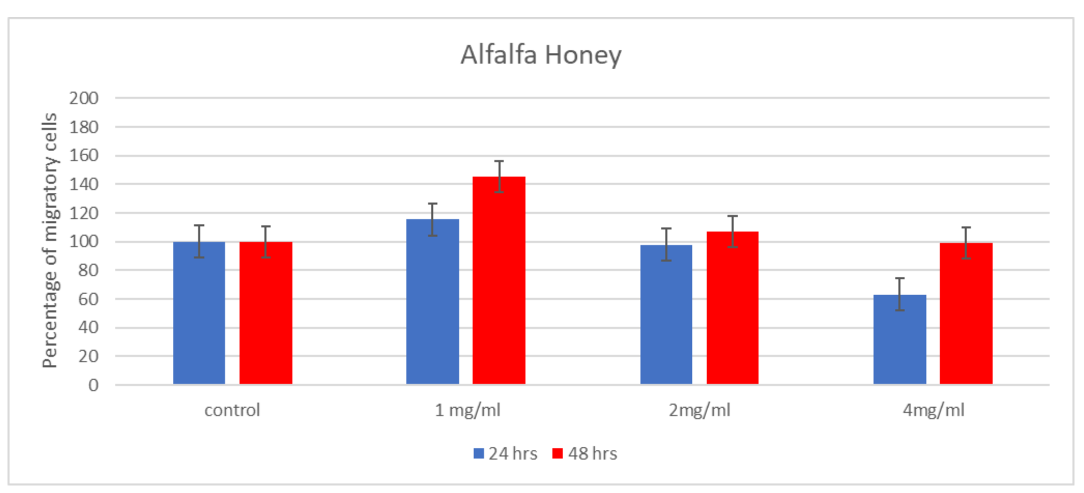

Figure 4.9: Quantification of wound closure as a percentage of migratory HaCaT cells during 24 and 48 hrs, for Alfalfa honey. Data are expressed as a percentage of untreated control (mean ± SD, n = 3). $p < 0.05$ vs control (unpaired Student's t-test).

4.3.3 Avocado Honey

Avocado honey was the most significant in promoting keratinocyte migration of all honeys examined. Figure 4.10 shows microscopy images from a scratch wound assay at different time points; the results are highlighted in Figure 4.11. Wound closure at 24 h ranged from 142% for 1 mg/mL to 177% for 2 mg/mL, with a clear concentration-dependent effect. At 48 h, closure was 152%, with 2 mg/mL causing complete closure.

Even the lower (1 mg/mL) and upper (4 mg/mL) doses caused high levels of wound closure, which is 182%, demonstrating that avocado honey significantly stimulates keratinocyte migration at all doses. These data, summarized in Table 4.7, suggest a strong, immediate effect on wound healing, particularly at 2 mg/mL, which may be the optimal concentration to induce migration.

Table 4.7: Effect of Avocado honey on HaCaT keratinocyte migration in the scratch wound-healing assay at 24 and 48 hours. Values are expressed as % of control (mean ± SD, n = 3).

Avocado Honey concentration (mg/mL)	24 hrs (% migratory cells) ± SD	48 hrs (% migratory cells) ± SD
Control	105 ± 10	100 ± 8

1	140 ± 10	150 ± 10
2	175 ± 10	185 ± 12
4	105 ± 8	115 ± 9

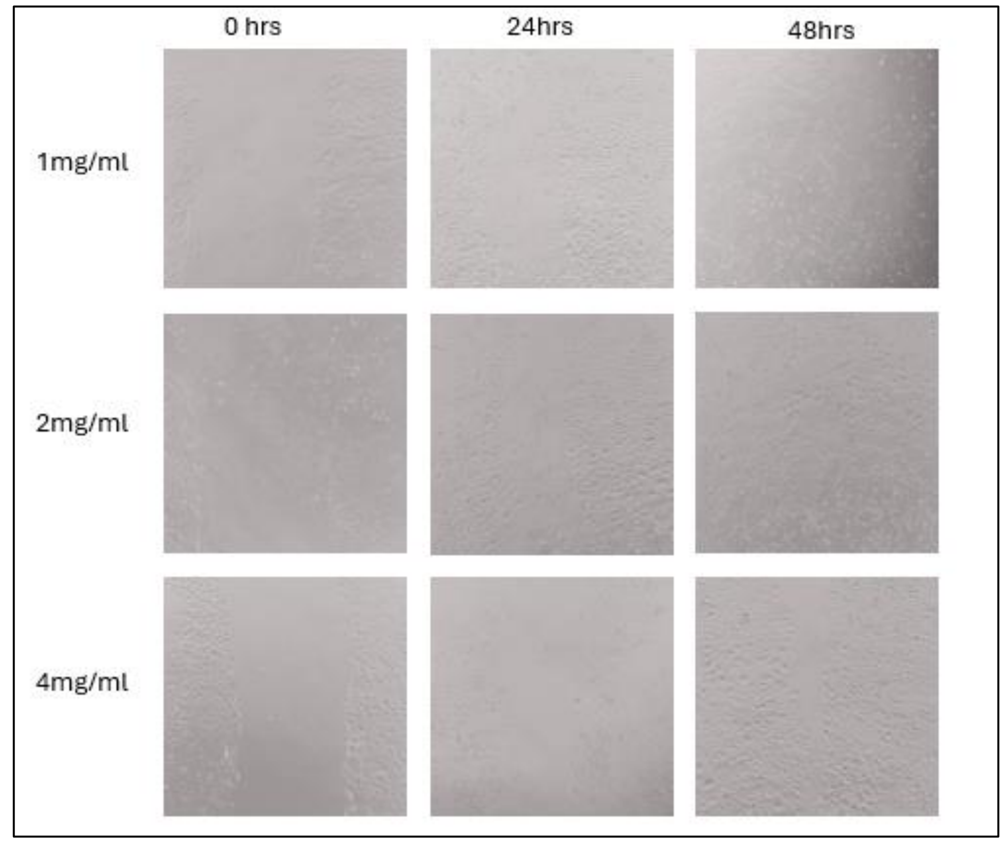

Figure 4.10: Representative micrographs from the scratch wound-healing assay of HaCaT keratinocytes treated with avocado honey. Images were taken at 0, 24, and 48 hours after scratch creation.

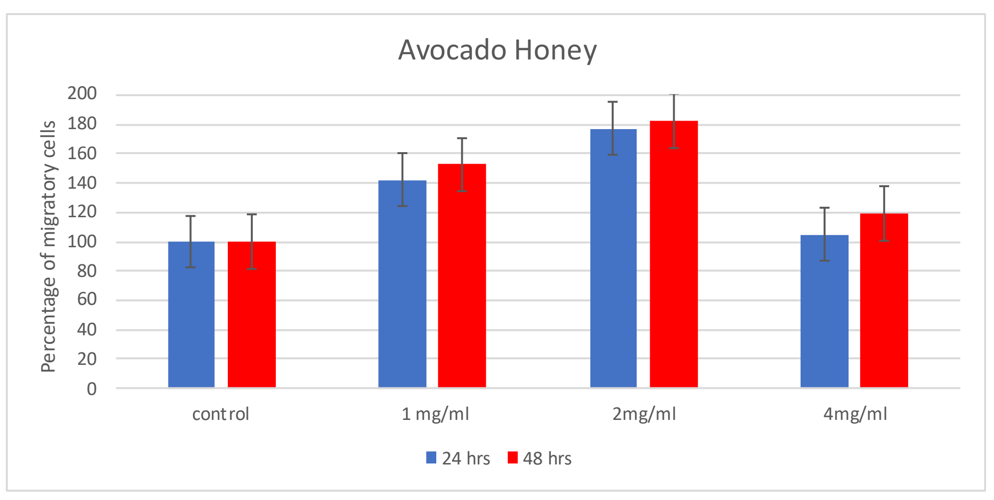

Figure 4.11: Quantification of wound closure as a percentage of migratory HaCaT cells during 24 and 48 h, of Avocado honey. Data are expressed as a percentage of untreated control (mean ± SD, n = 3). $p < 0.05$ vs control (unpaired Student's t-test).

4.3.4 Manuka Honey

Manuka honey also promoted extensive wound closure. Figure 4.12 shows microscopy images from a scratch wound assay at different time points; the results are highlighted in Figure 4.13. The closure reached 130% at 1 mg/mL at 24 h, with a little reverse concentration effect at the initial time point, where it decreased to 120% at 2 mg/mL and 86% at 4mg/mL. At 48 h, closure was 182%–108%, with the highest rate at 1 mg/mL.

These results, as summarized in Table 4.8, indicate that manuka honey enhances keratinocyte migration within a time period, with reduced doses particularly effective at maximum wound closure. Manuka honey tended to induce early and late-phase migration, which indicates great potential for wound healing.

Table 4.8: Effect of Manuka honey on HaCaT keratinocyte migration in the scratch wound-healing assay at 24 and 48 h. Values are expressed as % of control (mean ± SD, n = 3).

Manuka Honey concentration (mg/mL)	24 h (% migratory cells) ± SD	48 h (% migratory cells) ± SD
Control	105 ± 10	100 ± 8
1	130 ± 10	185 ± 12
2	120 ± 9	165 ± 10

4	85 ± 8	110 ± 9

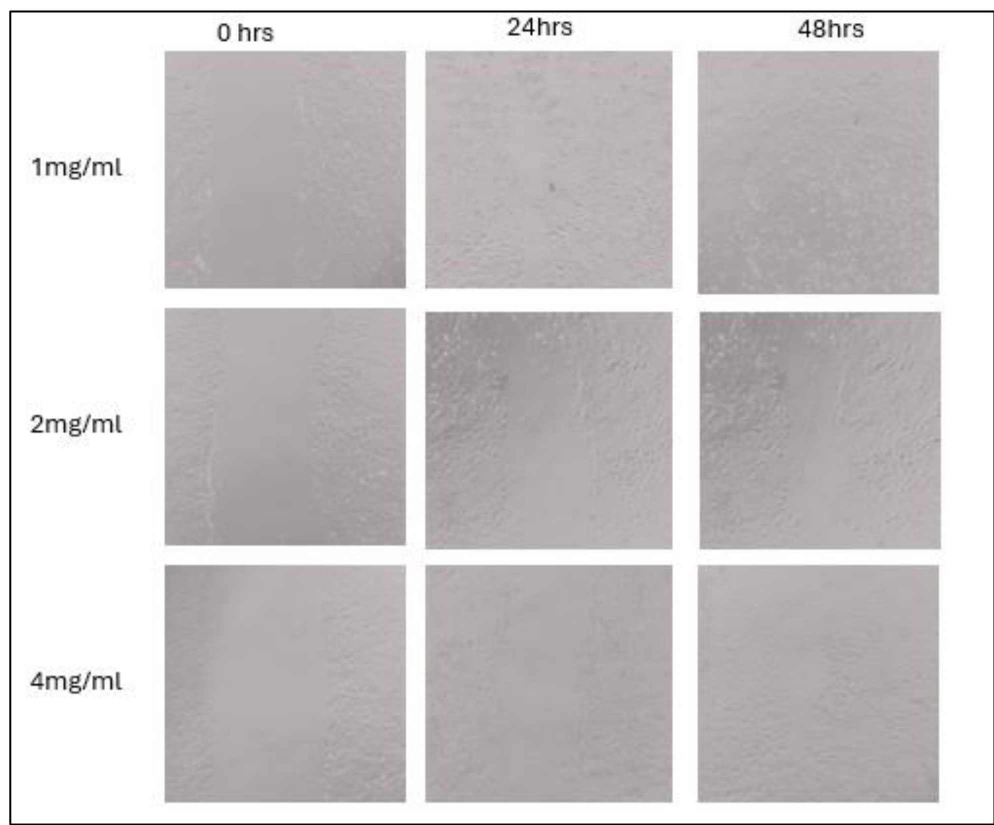

Figure 4.12: Representative micrographs from the scratch wound-healing assay of HaCaT keratinocytes treated with Manuka honey at different concentrations (1, 2, and 4 mg/mL). Images were taken at 0, 24, and 48 hours after scratch creation.

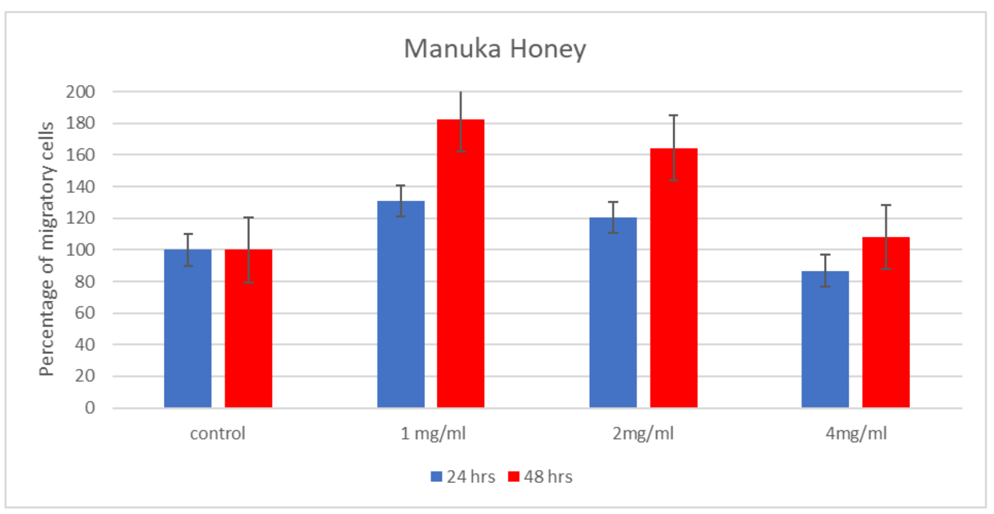

Figure 4.13: Quantification of wound closure as a percentage of migratory HaCaT cells during 24 and 48 h, for Manuka honey. Data are expressed as a percentage of untreated control (mean ± SD, n = 3). $p < 0.05$ vs control (unpaired Student's t-test).

4.3.5 Wound Scratch Assay Summary of Results

Wound scratch assay was used to test the impacts of Citrus, Alfalfa, Avocado, and Manuka honeys on HaCaT keratinocyte migration. The treatment was applied at 1, 2, and 4 mg/mL concentrations, and at 24 h and 48 h wound closure after treatment. The results indicate that all honeys positively affected keratinocyte migration, though the magnitude and direction varied according to honey type, concentration, and time.

- Citrus Honey:

  Citrus honey had a moderate stimulatory effect on keratinocyte migration. Wound healing was maintained at approximately 100% at 24 h for 1 mg/mL and 4 mg/mL, indicating early-stage activity. Wound healing was augmented at 48 h, from 113% to 127%, with maximum closure being at 4 mg/mL. These results point to Citrus honey furthering progressive cell migration over time.

- Alfalfa Honey:

Alfalfa honey showed a higher level of stimulation than Citrus honey. Wound closure after 24 h reached a peak at 115% in the 1 mg/mL concentration, with higher concentrations showing slightly lower activity, suggesting an optimal range for migration. At 48 h, closure also increased further to 145% at 1 mg/mL, proving an enduring stimulatory effect with time.

- Avocado Honey:

Amongst all honeys used, Avocado honey proved to have the strongest effect on keratinocyte migration. Wound closure at 24 h was between 142% for 1 mg/mL and 177% for 2 mg/mL, and there was a well-demonstrated dose-dependent effect. At 48 h, closure was 152% to 182%, and the 2 mg/mL concentration was near complete wound closure. Even the lower (1 mg/mL) and higher (4 mg/mL) concentrations had significant migration, demonstrating a strong and immediate stimulus to wound healing.

- Manuka Honey:

Manuka honey also promoted keratinocyte migration, but the response had a more reverse pattern. At 24 h, there was wound closure of 130% at 1 mg/mL, which decreased at 2 mg/mL (120%) and 4 mg/mL (86%), indicating an inverse concentration response at the start. At 48 h, closure was between 108% and 182%, with peak closure at 1 mg/mL. This suggests that lower concentrations of Manuka honey are more efficient at triggering early and late-phase migration, showing its potential as a potent stimulator of wound healing.

4.4 Production of Nitric Oxide -Griess Assay

The effect of different honeys on nitric oxide (NO) production in THP-1 cells was assessed by the Griess assay after 72 h of incubation.THP-1 monocytes (50,000 cells/2 mL,

24-well plates) were incubated with 2 mg/mL of each honey after stimulation with 1mg/mL LPS. NO levels were quantified in cell culture supernatant. The values are expressed as a percentage of unstimulated control (unstimulated cells without LPS) in Table 4.9.

LPS treatment induced a high level of NO production compared to the negative control, indicating successful inflammatory activation of THP-1 cells. Honey treatments were found to have variable effects, depending on the type of honey and pH, ranging from nearly complete inhibition of the LPS effect to partial induction of NO. Each honey type has data as indicated below (Figures 4.14-4.17).

Table 4.9: Effect of different honeys on nitric oxide (NO) production in LPS-stimulated THP-1 cells (% of unstimulated control, mean ± SD, n=3).

Sample	**% of control +/- SD**
Control (cells –LPS)	100
LPS (positive control)	370 ± 50
Citrus	250 ± 35
Alfalfa	110 ± 15
Avocado	100 ± 12
Manuka	296 ± 40

LPS stimulation (positive control) caused robust induction of NO release to 370 ± 50% of control. The robust increase certified the susceptibility of THP-1 cells to pro-inflammatory stimulation and the utility of the assay as a valuable marker of NO modulation.

4.4.1 Citrus Honey

Citrus honey decreases the production of NO to 250 ± 35% over control levels. This is still below the very high levels stimulated by LPS. Results shown in Figure 4.14.

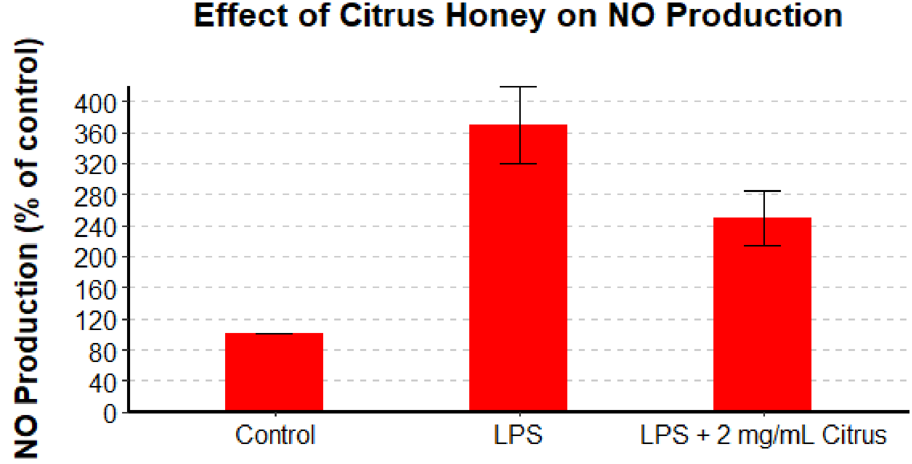

Figure 4.14: Effect of 2 mg/mL Citrus honey on nitric oxide (NO) production in LPS-stimulated THP-1 cells. Values are expressed as % of unstimulated control (mean ± SD, n=3). $p < 0.05$ vs LPS-treated control (unpaired Student's t-test).

4.4.2 Alfalfa Honey

Treatment with Alfalfa honey lowered the NO rate of production to 110 ± 15%, which is very close to the baseline, presenting a strong anti-inflammatory effect. Figure 4.15 highlights these values.

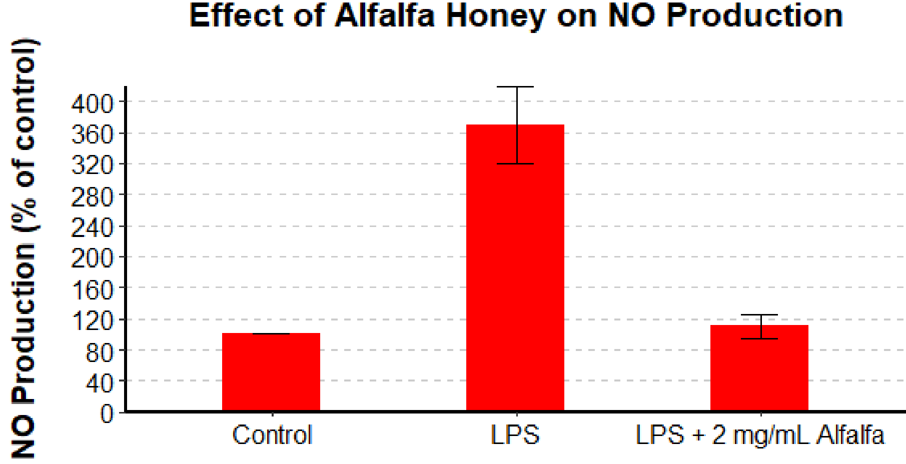

Figure 4.15: Effect of 2 mg/mL Alfalfa honey on nitric oxide (NO) production in LPS-stimulated THP-1 cells. Values are expressed as % of unstimulated control (mean ± SD, n=3). $p < 0.05$ vs LPS-treated control (unpaired Student's t-test).

4.4.3 Avocado Honey

On the other hand, Avocado honey generated an NO value the closest to the unstimulated control (100 ± 12%), suggesting a null effect on basal NO production. This suppression suggests that Avocado honey does have an anti-inflammatory effect on stimulated macrophages. Figure 4.16 highlights these values.

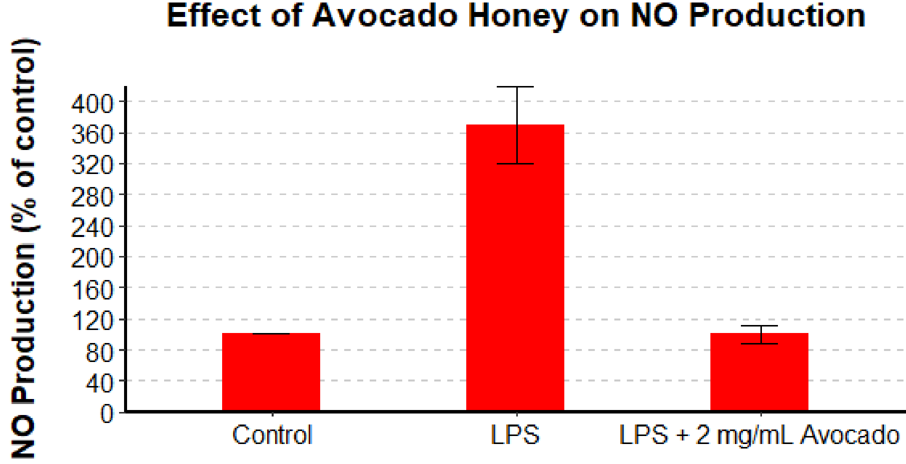

Figure 4.16: Effect of 2 mg/mL Avocado honey on nitric oxide (NO) production in LPS-stimulated THP-1 cells. Values are expressed as % of unstimulated control (mean ± SD, n=3). $p < 0.05$ vs LPS-treated control (unpaired Student's t-test).

4.4.4 Manuka Honey

Manuka honey showed increased values in nitric oxide compared to other honeys, reaching 296 ± 40% of control levels, suggesting that Manuka honey may contribute to defense against antimicrobial agents alongside a less pronounced pro-inflammatory role. Figure 4.17 highlights these values.

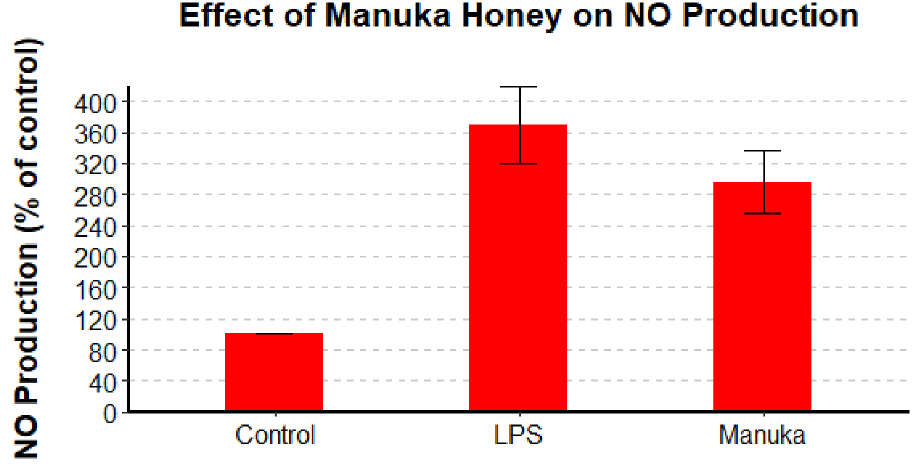

Figure 4.17: Effect of 2 mg/mL Manuka honey on nitric oxide (NO) production in LPS-stimulated THP-1 cells. Values are expressed as % of unstimulated control (mean ± SD, n=3). $p < 0.05$ vs LPS-treated control (unpaired Student's t-test).

4.4.5 Summary of Nitric Oxide Production – Griess Assay

The Griess assay showed that Citrus, Alfalfa, Avocado, and Manuka all showed variations in NO production. A positive control using LPS-treated cells showed a robust inflammatory stimulus (370 +/- 50% of base NO), while unstimulated cells represented the background NO level (100%).

- Citrus Honey:

LPS-stimulated NO was only 250 +/- 35% of the control level, indicating that it only partially inhibits this inflammatory response. This modulation may be due to phenolic compounds or some other bioactive content.

- Alfalfa Honey:

The Alfalfa honey not only tamp down NO release (to 110 +/- 15%), indicating strong suppression of inflammatory response under these conditions.

- Avocado Honey:

NO levels fell to normal (100 +/- 12%)—much less than when LPS is given alone, indicating a high anti-inflammatory effect under these conditions, reflecting efficient suppression of certain pathways for inflammation.

- Manuka Honey:

LPS-stimulated NO production is decreased to 296 +/- 40%. Characteristics like these may be due to its unique bioactive components, such as methylglyoxal and specific phenolics from the beehive, which inhibit somewhat extreme excretion of NO while still retaining antimicrobial effect.

4.5 External assays

4.5.1 Preliminary Antimicrobial Testing

Citrus, Alfalfa, and Avocado honeys also manifested other antibacterial activities on the four test microorganisms. Lower IC50, MIC, or MBC values indicate more activity.Table 4.10 provides a summary of the results.

Table 4.10: Antimicrobial activity of Citrus, Alfalfa, and Avocado honeys against four bacterial strains. MIC, MBC, and IC50 values are expressed as w/w% (mean ± SD, n = 3 independent experiments, four replicates each).

Kind of Honey	**IC50 (w/w%)**	**Haemophilus influenzae (w/w%)**		**Streptococcus pneumoniae (w/w%)**		**Klebsiella pneumoniae (w/w%)**		**Bacillus subtilis (w/w%)**	
		MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Citrus	10	3.125	.25	1.562	1.562	0.781	0.781	1.562	3.125
Alfalfa	0.30	12.5	25	6.25	6.25	3.125	6.25	3.125	3.125

Avocado	0.19	25	25	6.25	6.25	3.125	6.25	0.195	0.390

At IC50, Avocado (0.19) and Alfalfa (0.30) are considerably lower (more active) than Citrus (10). Looking at MICs: Citrus has the lowest MICs for H. influenzae (3.125), S. pneumoniae (1.562) and K. pneumoniae (0.781), indicating the strongest inhibition of the said strains; on the other hand H. influenzae MICs in comparison to Alfalfa (12.5) and Avocado (25) are higher. Avocado is highly effective against B. subtilis (MIC 0.195, MBC 0.390), followed by Citrus (MIC 1.562, MBC 3.125) and then by Alfalfa (MIC 3.125, MBC 3.125). These differences reflect the botanical influence on honey's antimicrobial activity.

Results of the MIC, MBC for each honey type are highlighted in Fig. 4.18-4.20

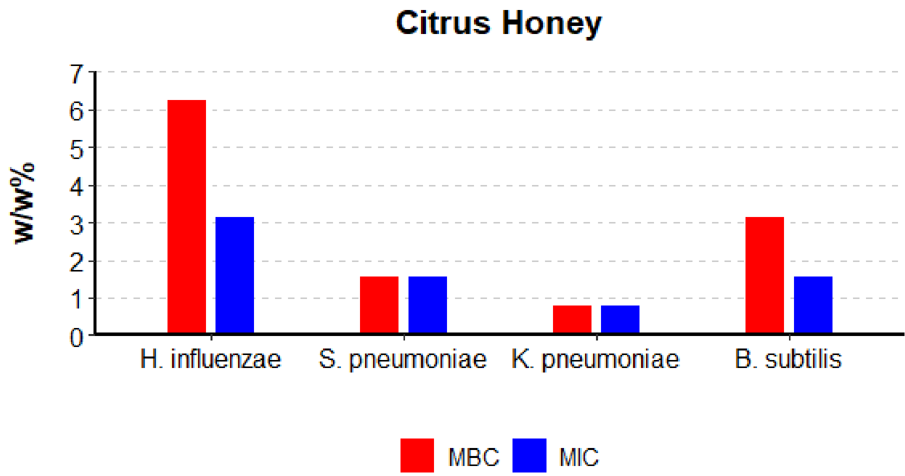

Figure 4.18: Antibacterial activity of Citrus honey against four bacterial strains. MIC and MBC values are expressed as w/w% (mean ± SD, n = 3 independent experiments, four replicates each).

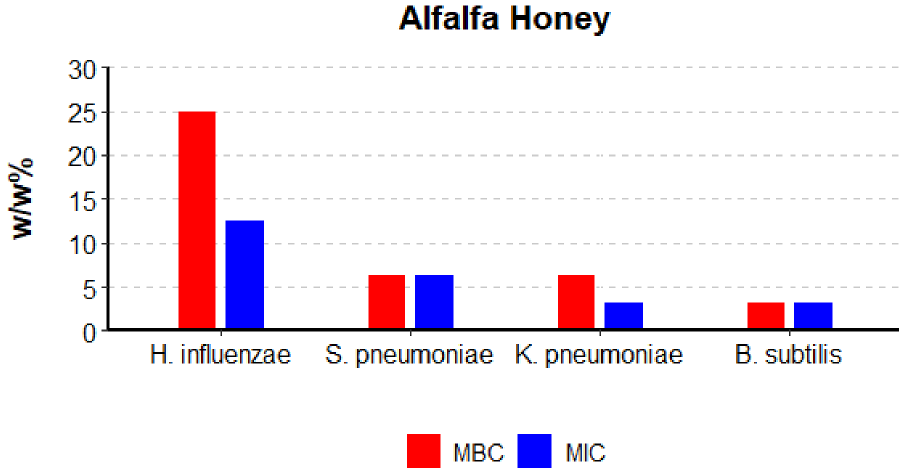

Figure 4.19: Antibacterial activity of Alfalfa honey against four bacterial strains. MIC and MBC values are expressed as w/w% (mean ± SD, n = 3 independent experiments, four replicates each).

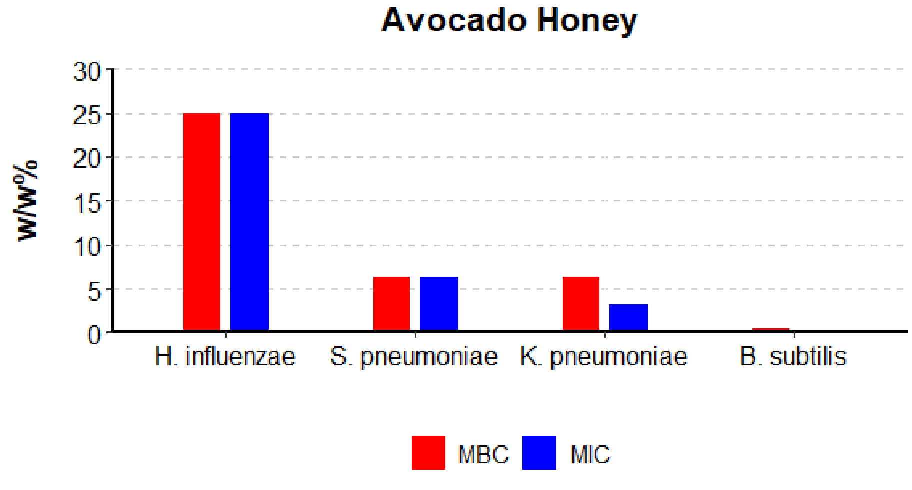

Figure 4.20: Antibacterial activity of Avocado honey against four bacterial strains. MIC and MBC values are expressed as w/w% (mean ± SD, n = 3 independent experiments, four replicates each).

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Antibacterial activity of Avocado honey against four bacterial strains. MIC and MBC values are expressed as w/w% (mean ± SD, n = 3 independent experiments, four replicates each).

4.5.2 Molecular docking

Due to time and resource limitations, molecular docking analysis was limited only to avocado honey, which, in all the in vitro experiments, consistently recorded the most promising results among the honeys tested. Avocado honey was therefore the subject of an in-silico docking approach to further elucidate the possible molecular reason for its bioactivity.

Table 4.11 provides calculated binding energies (Be, kcal·mol^{-1}) and inhibitory concentrations (Ic, µM) for six phenolics from avocado honey for four targets (3GRS — glutathione reductase; 4M6J — DHFR; 1N8Q — 5-LOX; 1XKK — EGFR). More negative Be and lower Ic are predictive of stronger binding and greater potency.

Table 4.11: Results of Docking Selected Avocado Honey Polyphenolic Compounds Against Target Proteins with Antioxidant, Antibacterial, Anti-Inflammatory, and Anticancer Properties.

Compound	**Antioxidant activity**		**Antibacterial activity**		**Anti-inflammatory activity**		**Anticancer activity**	
	3GRS		**4M6J**		**1N8Q**		**1XKK**	
	Be (Kcal/mol)	Ic (µM)	Be (Kcal/mol)	Ic (µM)	Be (Kcal/mol)	Ic (µM)	Be (Kcal/mol)	Ic (µM)
Gallic acid	-5.88	49.11	-5.93	45.22	-4.34	656.19	-4.94	240.70
p-hydroxybenzoic acid	-5.47	97.1	-5.99	40.2	-5.11	179.1	-5.40	109.2

Chlorogenic acid	-5.60	78.4	-6.58	15.2	-6.52	16.71	-5.11	180.9
Quercetin	-7.41	3.69	-7.42	3.65	-6.02	38.67	-6.87	9.19
Catechin	-6.67	12.4	-7.16	5.68	-7.15	5.72	-7.29	4.54
Galangin	-6.74	11.4	-7.19	5.41	-6.56	15.52	-7.27	4.69

Quercetin shows the best binding for the antioxidant target (3GRS) (Be = −7.41; Ic = 3.69 µM), followed by galangin and catechin, while gallic (−5.88; 49.11 µM) and p-hydroxybenzoic acid (−5.47; 97.11 µM) are weak.

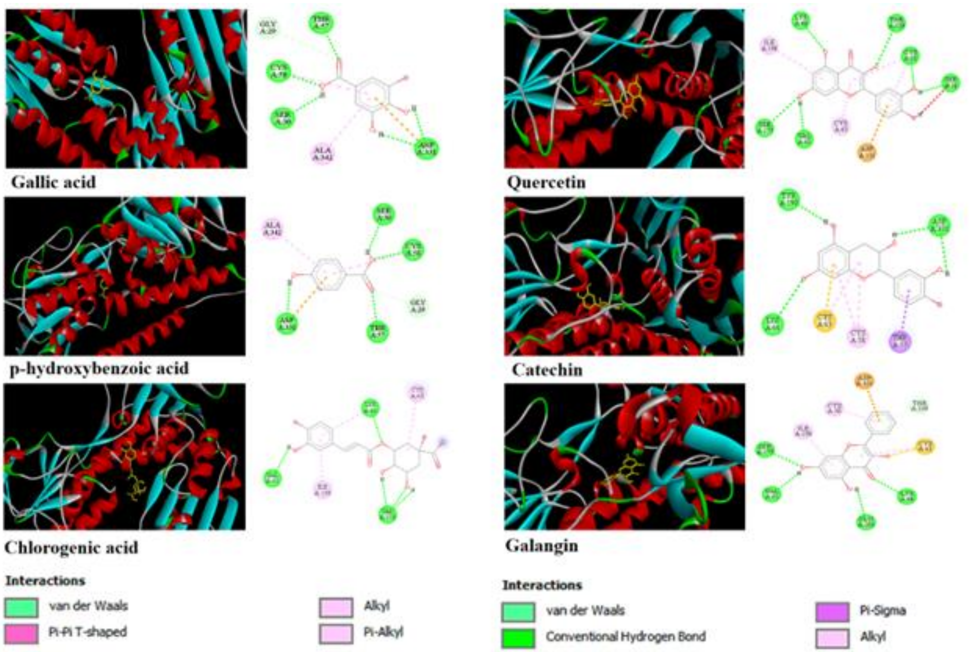

Figure 4.21:2D and 3D interactions of avocado honey phenolic compounds with glutathione reductase. The left panels are shown with protein surface and ligand; right panels provide interaction maps with hydrogen bonds, alkyl, and van der Waals contacts indicated by color.

Against antibacterial activity (4M6J) quercetin is again ahead (−7.42; 3.65 μM) with catechin (−7.16; 5.68 μM) and galangin (−7.19; 5.41 μM) close behind; gallic and p-hydroxybenzoic acids give much larger Ic values (45.22 and 40.62 μM).

Figure 4.22 shows that Quercetin exhibited the greatest predicted antibacterial binding with a dock score of −7.42 kcal·mol^{-1} and an estimated IC_{50} of 3.65 μM, which formed stabilizing contacts in the DHFR active site (e.g., Ala9, Val115, Thr60, Ile16). Catechin and galangin were also positively bound (binding energies −7.16 and −7.19 kcal·mol^{-1}, respectively) and engaged with conserved active-site residues contributing to substrate/cofactor recognition. Gallic acid and p-hydroxybenzoic acid were less well-bound and had higher estimated IC_{50} values, as would be expected for decreased predicted DHFR inhibition.

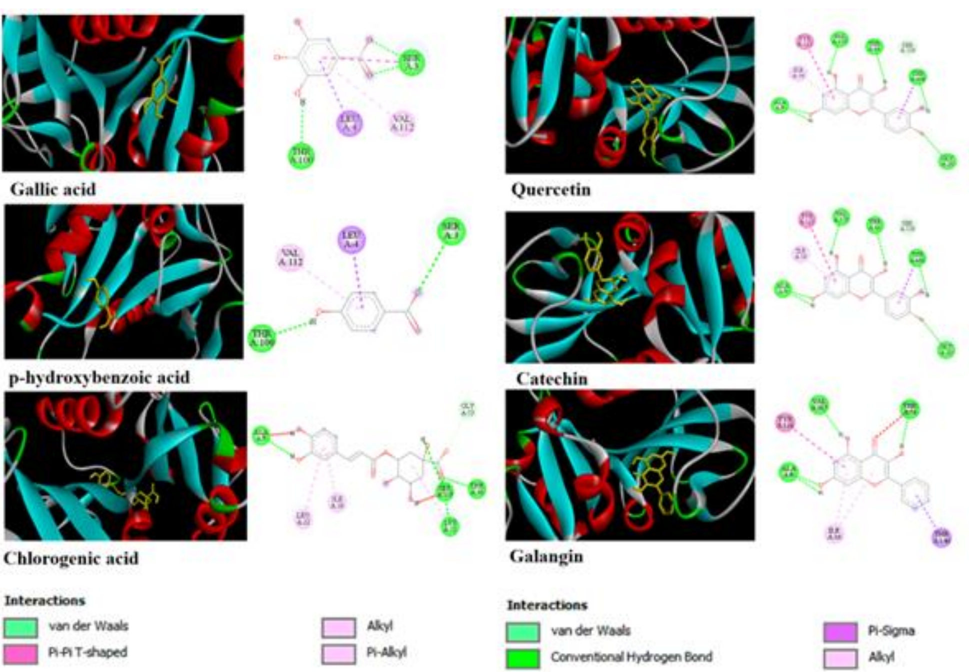

Figure 4.22: 2D and 3D interactions of Avocado honey phenolic compounds with bacterial dihydrofolate reductase. The left panels are shown with protein surface and ligand; right panels provide interaction maps with hydrogen bonds, alkyl, and van der Waals contacts indicated by color.

For 5-LOX (anti-inflammatory) catechin is the most active (Be = −7.15; Ic = 5.72 μM), with galangin (−6.56; 15.52 μM) and chlorogenic acid (−6.52; 16.71 μM) being moderately active; gallic acid and p-hydroxybenzoic acid are mildly active (Ic = 656.19 and 179.21 μM).

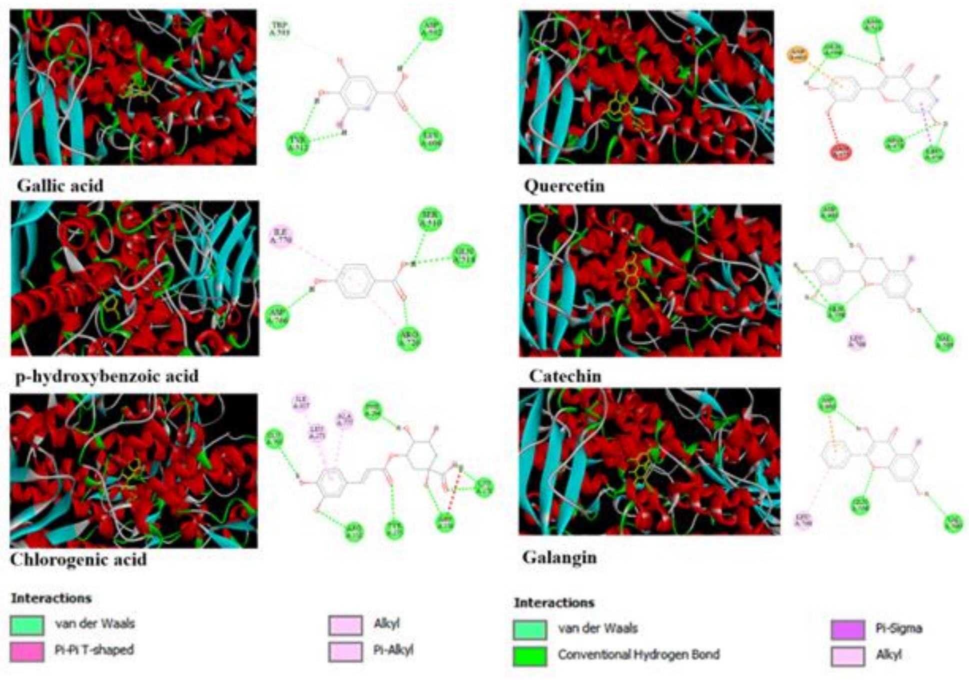

Figure 4.23:2D and 3D interactions of Avocado honey phenolic compounds with 5-lipoxygenase. The left panels are shown with protein surface and ligand; right panels provide interaction maps with hydrogen bonds, alkyl, and van der Waals contacts indicated by color.

For EGFR (anticancer) catechin (−7.29; 4.54 μM) and galangin (−7.27; 4.69 μM) have the lowest Ic values and best Be, while quercetin is also active (−6.87; 9.19 μM).

Antioxidant, Figure 4.21 illustrates representative 3D binding structures and 2D interaction maps of every phenolic compound docked into glutathione reductase (PDB ID: 3GRS). Quercetin, catechin, and galangin provided the broadest and most stable network of interactions, forming multiple representative hydrogen bonds and hydrophobic contacts with active-site residues CYS A:63, ASP A:331, LYS A:66, and SER A:30—features that are characteristic of strong stabilizing occupancy of the catalytic pocket.

anti-bacterial

As for anti-inflammatory, Figure 4.23 shows the six phenolics docking into 5-lipoxygenase (5-LOX; PDB ID: 1N8Q), an essential enzyme for leukotriene biosynthesis and inflammatory signalling. The strongest predicted LOX inhibitor was catechin (binding energy −7.15 kcal·mol^{-1}; estimated IC_{50} = 5.72 µM). Galangin and chlorogenic acid also showed favorable binding (binding energies −6.56 and −6.52 kcal·mol^{-1}; IC_{50}s ≈ 15–17 µM), and quercetin showed intermediate binding, and gallic acid and p-hydroxybenzoic acid

Figure 4.24 shows docking scores for the epidermal growth factor receptor (EGFR; PDB ID: 1XKK), a therapeutically important tyrosine kinase that is responsible for tumour cell survival and growth. Galangin was highly predicted to bind EGFR (binding energy −7.27 kcal·mol^{-1}; estimated IC_{50} ≈ 4.69 µM), while quercetin and catechin also showed high affinities (−7.29 and −6.87 kcal·mol^{-1}, respectively). Therefore, the docking results suggest quercetin, catechin, and galangin as putative EGFR binders deserving of follow-up biochemical evaluation.

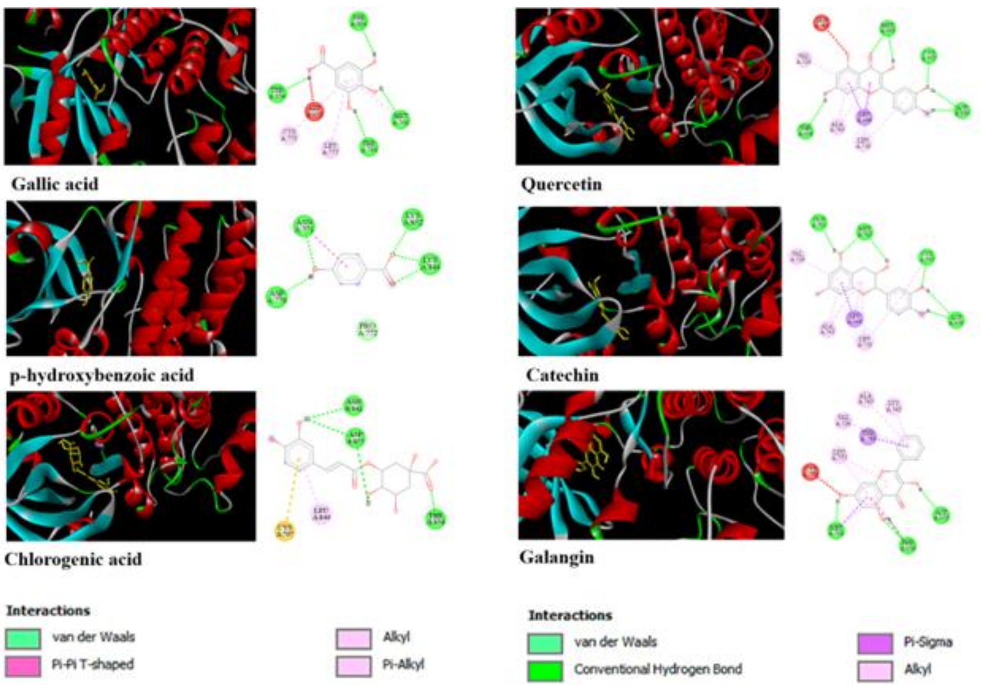

Figure 4.24: 2D and 3D interactions of Avocado honey phenolic compounds with the epidermal growth factor receptor. The left panels are shown with protein surface and ligand; right panels provide interaction maps with hydrogen bonds, alkyl, and van der Waals contacts indicated by color.

4.6 Summary of Results

Table 4.12 summarizes the effect of the honey samples tested.

Table 4.12: Comparative summary of the effects of different honeys on cell viability, wound closure, nitric oxide (NO) production, antimicrobial activity, and molecular docking.

Source	Endpoint	Citrus Honey	Alfalfa Honey	Avocado Honey	Manuka Honey
Laboratory results	Cell Viability	Slight increase	Increase at low dose, decrease at high dose	Strong increase at low dose, small decrease at high dose	Moderate increase at low dose, decrease at high dose
	Wound Closure	Low effect	High effect	Height effect	Moderate effect
	NO Production	Moderate decreases	Heigh decrease	Huge decrease	low decrease
External Lab	Antimicrobial Activity	Strain-selective.	Potent (broad but variable).	Most potent / Bacillus-targeting.	Not tested
	Molecular Docking	Not tested	Not tested	Support a molecular basis for avocado honey's antioxidant and antibacterial actions.	Not tested

Comparative summary of the effects of different honeys on cell viability, wound closure, nitric oxide (NO) production, antimicrobial activity, and molecular docking.

5 Chapter Five: Discussion

The Discussion below will be based on the results of a comparative biological assay, using in vitro models relevant to wounds. The main purpose of the current research was to evaluate the effect that different honeys have on keratinocytes and determine the final outcome of wound healing. For these purposes, the re-epithelialization capability was established through keratinocyte viability and migration, immune modulation was assessed through nitric oxide production as an index of immune homeostasis, while antibacterial activity was measured based on the ability of each honey to suppress the wound-associated bacteria.

5.1 Cellular Effects of Honey on Wound-Relevant Processes

Viability and migration results together suggest that honey concentration-dependently influences keratinocyte function. Though low concentrations maintained metabolic activity and facilitated migration, high concentrations disrupted wound closure, despite some survival (Iosageanu A. , Stefan, Craciunescu , & Cimpean, 2024). This implies that migration, relative to survival, is a more critical functional parameter, emphasizing the need for optimal dosing in wound repair.

5.1.1 Keratinocyte Responses to Honey Treatment: Viability, Proliferation, and Migration

All of the honeys had optimal proliferative activity at low concentrations (2–4 mg/mL), with increasing concentrations afterward either stabilizing or decreasing cell viability. This is a trend that suggests that there is an optimal range of concentrations for inducing keratinocyte proliferation, above which the effect is reversed. For Avocado, Alfalfa, and Manuka honeys, growth was significantly enhanced at 2 mg/mL, while Citrus honey responded less at all concentrations.

Although Citrus honey does not greatly accelerate keratinocyte migration but retains baseline closure rates. While a more pronounced effect was at 48 hours, with wound closure increasing progressively from 113% to 127%, the highest effect occurred at 4 mg/mL. This trend over time indicates that instead of stimulating an immediate, initial burst of migration, it stimulates a gradual increase in activity that is only discernible in the later phase. In general, it seems to induce long-term sustained migration but with lower potency than the other honeys tested.

Still, Citrus honey supported keratinocyte viability at levels greater than 53% for all concentrations, suggesting that its bioactive components exert their effects mainly to maintain cell viability and thwart oxidative stress rather than induce high levels of metabolic activity. This aligns with a study by (Li, Kandhare, Mukherjee, & Bodhankar, 2018). on hesperidin, a major citrus flavonoid, which has wound-healing activity primarily through angiogenesis by promoting VEGF-c, Ang-1/Tie-2, and TGF-β/Smad signaling. Since these are endothelial cell rather than keratinocyte cell processes, Citrus honey's induction of keratinocyte motility is comparatively restricted.

Alfalfa and Avocado honeys, intermediate with respect to phenolic content, exhibited a potent proliferative effect at low concentrations, with Alfalfa honey showing maximum closure at 24 hours of 115% was seen with the 1 mg/mL dose, although this action was less potent at higher concentrations. This trend of concentration suggests the existence of a spectrum of optimal stimulation in which intermediate levels are optimum for stimulating keratinocyte motility. At 48 hours, migration levels continued to increase, and 145% closure at 1 mg/mL was seen.

Both kaempferol and quercetin, present in alfalfa and avocado honey, modulate PI3K/Akt and MAPK/ERK pathways in dose-dependent mechanisms, as described in (Wani, et al., 2020); within the concentration ranges present in the 2–4 $mg \cdot mL^{-1}$ range, they can tip the signaling towards proliferation. Because total phenolics are moderate (not high), the shift to pro-oxidant/cytostatic activity at increased doses is delayed or dampened, and this explains the high peak stimulation with minimal inhibition at the highest dose.

Kaempferol and quercetin in moderate amounts can subtly amplify PI3K/Akt and MAPK/ERK signaling, focal adhesion turnover, and actin reorganization that underlie the

early migratory burst at 24 h. The long-term effect at 48 h should reflect continued mitochondrial protection and mild modulation of ECM remodeling. However, because total phenolics are intermediate, higher doses progressively impose osmotic or mild pro-oxidant stress and cause the observed loss in efficacy at high concentrations.

A study by (Yao, et al., 2014) demonstrated kaempferol inhibition of SUV-induced skin carcinogenesis provides mechanistic support for our model with phenolics from alfalfa. irradiation activates MAPK cascades in skin cells, such as ERK-mediated phosphorylation of downstream kinases RSK2 and MSK1, which ultimately regulate transcription factors regulating proliferation, survival, and tumorigenesis.

While the SUV study highlights chemopreventive inhibition during stress, our alfalfa model highlights a hormetic effect where low concentrations stimulate proliferation and greater concentrations eventually lead to cytostatic or pro-oxidant effects. Together, these findings suggest that kaempferol is able to specifically modulate MAPK/ERK signaling in skin cells in such a manner that the net response is a function of cellular environment and concentration, with a biphasic mechanism for stimulation of alfalfa phenolic-treated keratinocyte growth.

Amongst all honey samples tested, Avocado honey exhibited the strongest stimulation of keratinocyte migration. Wound closure rates after 24 hours were already substantial, ranging from 142% at 1 mg/mL to 177% at 2 mg/mL. Notably, the 2 mg/mL treatment resulted in almost full closure of the wound gap within the time period of 48 hours, showing its very high efficacy. At 48 hours, closure values ranged from 152% to 182%, and again, the optimal concentration remained 2 mg/mL. At the higher concentration (4 mg/mL), migration was also potent, demonstrating that Avocado honey remained highly stimulating across a broad range of doses.

Avocado's high epicatechin and ferulic/protocatechuic acid composition can strongly maintain cellular redox homeostasis and mitochondrial competence at low levels, promoting ATP synthesis and proliferation. Epicatechin and analog flavanols are also reported to enhance endothelial/epithelial survival and sensitization of cells to growth factor signaling. As Avocado possesses a high but not excessive amount of phenolics, it causes firm

stimulation at therapeutic low doses but tends towards inhibitory action when in higher concentrations because the phenolic load is stronger.

These findings are consistent with studies on Persea americana leaf extract by (Rosa, et al., 2025) , where key flavanols such as epicatechin, catechin, and quercetin were identified as active compounds that promote wound healing primarily through NF-κB1 inhibition. The molecular mechanisms revealed by network pharmacology and molecular docking corroborate the idea that these flavanols can optimize cellular energetics and signaling pathways, sustaining wound closure processes. Together, these data support the notion that Avocado-derived flavonoids effectively balance stimulatory and protective actions, enhancing motogenic responses in keratinocytes while avoiding cytotoxicity

As for Manuka honey, suppressed viability most profoundly at high doses, particularly at 16 mg/mL, indicating that it may induce cytostatic or mild cytotoxic effects when utilized at high concentrations. The dose-dependent tendencies observed here are in agreement with previously published literature, which indicates that honey has both inhibitory and proliferative effects on mammalian cells, controlled to a large degree by its chemical composition and the concentrations employed.

Manuka honey exhibited a more complex, concentration-related pattern. After 24 hours, migration was strongly stimulated from 1 mg/mL to 130% closure. The effect decreased with higher concentrations, where it reached 120% and 86% closures at 2 mg/mL and 4 mg/mL, respectively. This would suggest an inverse concentration-response relationship in the initial phase, where lower doses were most effective and higher doses potentially inhibitory.

At 48 hours, migration increased in all concentrations, from 108% to 182%, with once more the greatest effect observed at 1 mg/mL. This implies that Manuka honey can induce both early and late-stage keratinocyte migration but only over a narrowly optimal concentration range. Increased concentration may introduce inhibitory effects, likely owing to excessive concentration of methylglyoxal (MGO) and other reactive constituents unique to Manuka honey.

5.1.2 Mechanistic considerations underlying viability and migration

5.1.2.1 Botanical source effect

These study results strongly agree with recent findings by (Iosageanu A. , Stefan, Craciunescu, & Cimpean, 2024), who investigated phenolic acid content and its skin permeation through honeys of differing botanical origin. confirming that the floral source determines the kind and amount of phenolic acids present, and that they can penetrate and be deposited in the skin efficiently, especially when formulated into hydrogels. Simultaneously, the study (Nowak, et al., 2025), demonstrated that phenolic content is strongly correlated with enhanced biological activities, including keratinocyte proliferation, fibroblast collagen production, and regulation of inflammatory cytokines.

5.1.2.2 Matrix remodeling and chemotactic signaling

Besides intracellular signaling, honeys can influence extracellular matrix (ECM) remodeling and chemotactic signals. Polyphenols and trace sugars are capable of controlling MMP expression and cytokine gradients guiding directional migration. The distinction between antioxidant protection and controlled redox signaling will determine whether the effects are motogenic or become inhibitory by oxidative stress at higher concentrations (Majtan, et al., 2013). These observations are supported by recent findings highlighting honey's robust antioxidant capacity, which is mediated by its phenolic acids, flavonoids, enzymes, and other bioactive compounds (Tlak Gajger, Dar, Ahmed, Aly, & Vlainić, 2025)

The synergistic activity of these constituents not only neutralizes excessive ROS but also regulates redox-sensitive signaling, thereby providing controlled antioxidant protection. This mechanism helps explain why low-to-moderate honey concentrations can promote motogenic effects through ECM remodeling and directional migration, while higher concentrations risk inhibitory outcomes due to oxidative stress. The study underscores that honey's composition—particularly its polyphenolic and enzymatic content—critically dictates its ability to balance cellular redox signaling and support wound healing.

5.1.3 Practical interpretation

The MTT findings suggest that honey's regenerative benefit depends on reproducing physiological, not maximal stimulation of keratinocyte metabolism, where keratinocyte viability and proliferation are enhanced without inducing oxidative or osmotic stress. Clinically, this suggests that honey-based formulations should be diluted to deliver comparable sub-cytotoxic concentrations at the wound surface, rather than being applied in their undiluted form.

Incorporating honey into hydrogel or film dressings would allow controlled release within this beneficial range, sustaining metabolic activation during the early proliferative phase while avoiding mitochondrial suppression at higher local doses. As demonstrated in (Yasin, Said, Halib, Rahman, & Mokhzani, 2023) honey-loaded hydrogels produce controlled, long-lasting release of bioactive constituents, create an environment of moist wound, and enhance the growth of keratinocytes and re-epithelialization.

The migration data emphasize that lower honey concentrations, which are more optimal for proliferation, should be used for formulations intended for the early re-epithelialization phase. Controlled-release matrices that provide gentle, sustained delivery can replicate the beneficial signaling conditions observed in vitro, enhancing keratinocyte migration without provoking oxidative stress or membrane disruption. This implies that dressing formulations should be phase-specific, with concentration and release rate adapted to whether the therapeutic goal is to stimulate migration or proliferation (Wang, et al., 2025).

5.2 Effect of Honey on Inflammatory Modulation

Avocado and Alfalfa honeys provided the strongest anti-inflammatory pattern. Avocado reduced NO from 370 ± 50% to 100 ± 12% (no different from unstimulated control) and Alfalfa to 110 ± 15%, reducing nitrite accumulation to nearly baseline levels despite LPS challenge. These findings show that both honeys strongly inhibit the LPS-induced NO response under the used conditions.

In a study conducted by (Kim, et al., 2018), The Acacia-honey data (AH) provide a mechanistic basis for our NO findings because AH suppressed NF-κB activation and iNOS expression caused by LPS in RAW264.7 macrophages, consistent with our results. This partially explains why Avocado and Alfalfa produced near-baseline levels of nitrite in our experiments: their phenolic/flavonoid content (especially epicatechin, quercetin/kaempferol families) is reported to suppress TLR4 to NF-κB signaling and thereby iNOS transcription (Afrin, et al., 2018), and may further suppress NO enzymatic production.

An experiment about Avocado (Avocado soybean unsaponifiables) by (Au, Al-Talib, Au, Phan, & Frondoza, 2007) , demonstrates that bioactive extracts are able to significantly suppress pro-inflammatory mediators, TNF-α, IL-1β, COX-2, and iNOS, both in chondrocytes and monocyte/macrophage-like cells, with correlated reductions in released PGE_2 and nitrite. This is similar to Avocado honey, where the strong inhibition of NO reflects either a higher local concentration of phenolic inhibiting species or the presence of other scavenging/enzymatic fractions. Functionally, as ASU normalizes hyper-inflammatory signaling, Avocado honey might also modulate pathological iNOS overactivity in keratinocytes, providing a protective effect in chronic or non-healing wound conditions by preventing sustained nitrosative stress and promoting controlled migration and repair.

Citrus honey had a moderate effect ($250 \pm 35\%$ of control), it inhibited but did not abolish the LPS response. The incomplete inhibition suggests that Citrus honey contains active constituents that suppress inflammatory signaling.

As for citrus honey, a study by (Kim, Kim, Boo, Kim, & Kim, 2023) demonstrated the effect on LPS-stimulated RAW 264.7 cells, the ethyl acetate fraction inhibited NO production, iNOS expression, and inflammatory cytokines by downregulating NF-κB and MAPK signaling in a dose-dependent manner. Our study's moderate NO reduction finding concurs with the partial NF-κB/iNOS activity inhibition in the EtOAc fraction, highlighting the importance of composition in anti-inflammatory activity. Citrus honey exerted an intermediate effect on keratinocyte NO generation, consistent with weaker NF-κB/iNOS inhibition, likely due to relatively lower phenolic content. This suggests that bioactive phytoconstituents from Citrus fruits or honey can influence inflammatory signaling,

Manuka honey produced the greatest NO among honeys tested (296 ± 40%), approaching but not surpassing the LPS positive control. The presence of unique Manuka constituents (e.g., methylglyoxal and specific phenolics) may be responsible for antimicrobial activity and support a sustained or elevated NO production.

Manuka's sustained high NO most likely reflects incomplete inhibition of NF-κB and/or alternative/distal mechanisms ensuring iNOS activity (Chang, Wang, & Wu, 2005), found that Methylglyoxal (MGO) increases nitric oxide (NO) and superoxide (O_2^-) in vascular smooth muscle cells, generating sustained levels of NO and reactive oxygen species.

5.3 Dose-dependent antimicrobial effects (IC_{50}, MIC, MBC)

The experimental data demonstrate two complementary but apparently conflicting potency profiles. For the cumulative inhibitory measure assayed (IC_{50}, w/w%), Avocado (0.19) and Alfalfa (0.30) are significantly more inhibitory than Citrus (10.00)—a ~50× contrast of Avocado to Citrus and ~33× contrast of Alfalfa to Citrus.

This result indicates that, per mass in the IC_{50} assay format, Avocado and Alfalfa exert strong broad inhibitory pressure.

When focused on organism-specific endpoints (MIC/MBC). Citrus has the lowest MICs against Haemophilus influenzae (3.125), Streptococcus pneumoniae (1.562), and Klebsiella pneumoniae (0.781), outperforming both Alfalfa and Avocado by factors of ~4× to ~16×. In contrast, Avocado alone is very potent against Bacillus subtilis (MIC ≈ 0.195; MBC ≈ 0.390), with MICs ~8–16× lower than its competitors.

Alfalfa is in an intermediate position, having very low IC_{50}, which suggests broad potency, but its MICs are generally higher than Citrus against the respiratory/GI pathogens and higher than Avocado against B. subtilis and reflect a balanced though less organism-selective antibacterial profile. This pattern suggests a mix of broad-acting mechanisms, such as H_2O_2, moderate burden of phenolics (Naskar, et al., 2024) .

Alfalfa honey constitutes a mid-range profile, integrating modest total phenolics and hydrogen peroxide production to apply wide but balanced antibacterial pressure. Such

composition and structure interactions account for the 4–16× MIC range apparent between species and honeys, as pointed out in a recent study on alfalfa honey by (Bravo & Santhoff, 2024) Alfalfa honey has broad-spectrum antibacterial activity capable of clearing infection, including antibiotic-resistant types like MRSA, without relying on highly potent species-specific molecules. This aligns with our observation that Alfalfa honey applies wide but moderate antibacterial pressure, as supported by the 4–16× MIC range against a diversity of bacterial species.

These quantitative differences have clear functional implications. 4×–16× MIC value variations are far from a trivial laboratory phenomenon; they reflect clinically relevant differences in the concentrations at which pathogens can be suppressed or eliminated. In topical preparations, where concentrations achievable are narrow and must not induce host cytotoxicity, the difference between a honey having an MIC of 0.78 and a honey having an MIC of 3.12 can be the difference between effective microbial suppression and failure of therapy.

Avocado's superior MIC/MBC pair for B. subtilis strongly suggests a bactericidal effect at low levels against that bacterium, an attractive benefit when rapid kill is needed. In contrast, Citrus's extremely low MICs against the respiratory/GI pathogens tested can suggest novel phytochemical preferences for biochemical targets of those bacteria and make Citrus a leading candidate when those organisms are suspected.

The strong bactericidal effect of Avocado honey on B. subtilis also aligns with recent reports from Persea americana peel extracts (Trujillo-Mayol, Casas-Forero, Pastene-Navarrete, Lima Silva, & Alarcón-Enos, 2021) that indicated measurable inhibition of Gram-positive bacteria, including Bacillus cereus, in various extract fractions. Fractions richest in phenolics and proanthocyanidins (APE and the organic fraction) in that study showed the strongest antibacterial and antioxidant activities, which supports the notion that phenolic compounds in avocado are accountable for antimicrobial activity.

Our observation of a potent MIC/MBC pair against B. subtilis thus agrees with the trend that phenolic-rich avocado matrices manifest stronger activities against spore-forming Gram-positive species. However, relative to the relatively higher MICs obtained for solvent extracts, the lower effective concentrations for Avocado honey suggest that the honey matrix

may enhance bioavailability or synergy among antioxidant and osmotic stress determinants to achieve a faster bactericidal effect. Taken together, these findings recognize avocado products as promising natural antimicrobials, with honey preparations likely to convey added efficacy through multi-component interactions.

As for Citrus honey, one of its most prominent flavonoid activity Rutin, showed a great effect against Klebsiella pneumoniae, a study gives us experimental evidence by (Wang, et al., 2021) Rutin was the most potent of 10 flavonoids tested for antioxidant activity and was effective in inhibiting K. pneumoniae growth (MIC = 1024 μg/mL) and biofilm development. Mechanistically, rutin repressed significant biofilm-associated genes, such as luxS and wabG, and mrkA expression was correlated with biofilm biomass. The findings concur with our observation that citrus honey, with only low total phenolic content, can confer particular antibacterial activities through the use of low-molecular-weight, bioavailable molecules, which are able to engage membrane and enzyme targets in targeted species.

These differences in antimicrobial properties are not to be considered inconsistent. On the contrary, this variance is evidence of the complementary antimicrobial properties of the honeys, which are being used to various purposes. While some honeys, such as Alfalfa and Avocado, have general inhibitory properties at lower concentrations, others, such as Citrus and Avocado, are more powerful and concentrated antimicrobials. This activity profile suggests that the antimicrobial actions of honey are based on either general factor like hydrogen peroxide and/or osmotic stress, and/or selective phytochemical interactions that could be used based on the intended therapy.

5.4 Molecular Docking: Mechanistic Insights from Avocado Honey Phenolics

This section reports the results of molecular docking studies carried out on some phenolic compounds identified in avocado honey. Because of time and cost constraints, this in silico analysis was made on avocado honey alone. However, this approach was scientifically rational as avocado honey showed greater efficacy in previous biological assays such as improved keratinocyte viability and migration rate, significant antibacterial properties, and modulation of inflammation compared to the other honey samples tested.

Hence, avocado honey was identified as a significantly important sample amenable to in silico or hypothesis-generating approaches to infer some molecular interactions in relation to redox modulation, inflammation, bacterial metabolism, and keratinocyte proliferation. Molecular docking analysis results should be considered carefully in conjunction with results from biological experiments and published literature to infer a molecular mechanism.

5.4.1 Glutathione Reductase (GR) Binding and Redox Modulation

Docking results revealed that quercetin, catechin, and galangin established stable binding interactions with glutathione reductase (GR) through the process of hydrogen bonding and hydrophobic interactions with specified active site residues CYS63, LYS66, and ASP331. The binding energies and inhibition constants for the docked complex of quercetin were approximated to be −7.41 and 3.69 μM, respectively.

These results support the findings of the study by (Güller, Karaman, Güller, Aksoy, & Küfrevioğlu, 2021) demonstrating the inhibition of the human GR by quercetin ($IC_{50} \approx 57.8$ μM) based upon docking interactions with CYS63 and LYS66. Consistent evidence also exists of the inhibition of quercetin upon the activity of GR in cell and enzyme-based assays (Lee, Jang, Kim, Oh, & Kim, 2023).Although the observed inhibition is focused upon an enzyme-based sub-system, the results of the present study propose the involvement of the biological phenomenon in the overall antioxidant complex of the honey matrix. Within this framework, the transient inhibition of the GR enzyme could be potentially mitigated or balanced by the presence of other members in the redox array of the honey matrix, potentially allowing the regulation of the redox signaling pathways rather than the inducement of oxidative stress.

5.4.2 Dihydrofolate Reductase (DHFR) Binding and Antibacterial Mechanism

Quercetin, catechin, and galangin had good predicted binding affinity to the conserved bacterial DHFR active site, with good interaction with the active site residues essential for the catalysis and binding of the inhibitor (Ala9, Ile16, Thr60, and Val115), with a predicted inhibition constant of about 3.65 μM for the Ic form of quercetin. This may provide a

mechanism for the observed potent bacteriostatic activity of avocado honey against Bacillus subtilis.

Inhibition of DHFR interferes with folate metabolism and nucleotide synthesis, a proven anti-bacterial approach, and the docking study provides molecular evidence supporting this action mechanism (Gallegos, Vargas, & Rodríguez-García, 2016) . The findings are in excellent agreement with the docking experiment reported by (Praveen, et al., 2023), who showed very good binding affinity of honey-derived phenolics with bacterial DHFR approaching those of known inhibitors. Based on the above findings, two mechanisms contribute to the anti-bacterial action of the presented avocado honey: Selective inhibition of the essential metabolic enzyme DHFR, together with non-specific action mechanisms such as membrane disruption, and induction of oxidative stress and hydrogen peroxide toxicity. Species-specific sensitivity, especially among Bacillus strains, could be due to variations in the requirement of folates or membrane characteristics and/or the ability.

5.4.3 5-Lipoxygenase (5-LOX) Interaction and Anti-Inflammatory Implications

Results from docking experiments revealed catechin as the top predicted binder of 5-lipoxygenase, along with galangin and chlorogenic acid. These compounds interacted through residues participating in substrate access and catalysis. This implied leukotriene biosynthesis inhibition.

Potential inhibition of leukotriene biosynthesis was suggested, as the interacting residues belonged to the substrate access and catalysis region in 5-Lip.

This mechanism offers a rationale for the attenuated production of nitric oxide in THP-1 cells treated with avocado honey in response to LPS stimulation that can be justified by the known amplificatory effect of leukotrienes on the NF-κB and iNOS pathways. Although the evidence for the inhibition of 5-LOX by catechins has been previously shown (Choi, et al., 2004), There has been very recent follow-up research that established the potent and selective inhibition of the human 5-LO enzyme by catechol compounds (Krauth, et al., 2023) by both the catalytic and the allosteric sites. The remarkable structural analogy between catechins

and 5-LOX inhibitors of the catechol type provides further support for the hypothesis regarding the anti-inflammatory effect of 5-LOX inhibition by the phenolic contents of avocado honey.

5.4.4 EGFR Binding and Biphasic Effects on Keratinocyte Proliferation

Catechin, Galangin, and Quercetin showed desirable predictions of binding to the ATP-binding site of Epidermal Growth Factor Receptor (EGFR) with binding energy between -7.3 kcal/mol and -6.9 kcal/mol involving major residues of kinases like MET793, THR854, and ASP855 of EGFR protein. These results indicate that weak concentration-dependent EGFR modulators could be present in Avocado Honey in the form of flavonoids.

This modulation addresses the biphasic nature of HaCaT cell MTT and scratch assay results, in which a low concentration of honey stimulated cell proliferation and migration, but higher concentrations inhibited these activities. A parallel EGFR binding profile of catechin compounds has been shown in computational studies reported by (Chen, Zhang, Dong, Liu, & Bai, 2025) in 2025, sustaining strong associations with both wild-type and mutant EGFR. Associated experimental data confirm the modulation of keratinocyte cell fate by quercetin, as an inhibitor of PI3K/AKT, Notch, and metabolism in a cell and tissue-dependent fashion, respectively by (Rehan, et al., 2024). All of these pieces of evidence contribute to EGFR as a putative target for the biphasic effect of avocado honey on HaCaT cells.

5.5 Integrative Interpretation: Honey-Based Wound Therapy

This chapter presents the experimental data on the use of honey in wound healing with the aim of providing phase-specific treatment based on evidence from the biological response associated with healing. In this case, by considering the data on keratinocyte viability and migration, NO expression, and antimicrobial properties, we provide justification on how different honey varieties should be utilized based on the type and phase of the injury.

5.5.1 Criteria for Phase-Specific Honey Selection

The current data show that monofloral honey samples have different or counteractive effects on inflammation, antimicrobial properties, and keratinocytes. Given the fact that the biological processes lead the various phases of the wound healing process, the above findings strongly support the idea of phase-specific therapy, rather than standardized treatments with honey.

Honey varieties used for different stages of the wound healing process were ensured through critical functional parameters derived from biological evaluations, which included regulation of nitric oxide (NO) production, antimicrobial properties, as well as cell viability/migration for keratinocytes. These parameters offer a mechanistic and relevant context for evaluating the performance of honey in wound healing.

Honeys that lower NO production strongly, such as Avocado, Alfalfa, are more suitable for chronic/inflamed wounds, while the other honeys that lower it Wang moderately, such as Citrus, Manuka, are considered potentially useful only for short-term, tightly controlled use in very contaminated wounds.

Thus, a two-stage dressing is recommended, the first is an initial short-release layer loaded with a Citrus/Manuka fraction (to promote early antimicrobial/immune activation), followed by a sustained-release Avocado/Alfalfa layer to reduce NO and support resolution. The Griess data define the timing and relative dosing for each layer — initial burst for 24–48 h, then maintenance release calibrated to avoid NO rebound (Kondej, et al., 2024).

The antimicrobial screen gives a pragmatic, pathogen-directed guideline for selecting and creating honey-based topical therapy. In short, utilize the antimicrobial information as an indicator of pathogen matching and as a formulation requirement. General low-IC_{50} honeys (Alfalfa, Avocado) are good generalist suppressors where mixed flora would be expected; (Citrus against selected Gram-negative respiratory/GI pathogens; Avocado specifically against Gram-positive Bacillus) are worth using when the likely pathogens are known or suspected.

When wound microbiology reveals Gram-negative respiratory/GI flora, topical Citrus-based preparations are an option to consider; when Gram-positive spillover/spore-formers are present, Avocado-enriched preparations would be the first choice. Alfalfa is an appropriate fallback for combinations or ambiguous flora.

MICs are practicable only if the dressing or gel can deliver and maintain high enough local concentrations without being toxic to host tissues. So, design controlled-release matrices (hydrogels, impregnated films, layered dressings) in vitro-calibrated to release in simulated wound fluid so that the released concentration at the wound interface equals the antimicrobial window but within keratinocyte safety margins established in the MTT/scratch data.

5.5.2 Citrus Honey: Early Immune and Antimicrobial Activation with Limited Proliferative Support

The low cytostatic effect shows that Citrus honey has a stable cellular state such that keratinocytes can survive and proliferate without being pushed prematurely into hyper-proliferation. While this lack of intense proliferative stimulation might at first blush be a liability, it may in fact be synergistic with the requirements of the inflammatory stage of wound healing, where regulated cellular activity is crucial to stage the events that follow.

A study by (Al-Waili, Salom,, & Al-Ghamdi, 2011) demonstrated honey's ability to reach a potent NO response, suggesting that it can enhance innate immune function at the wound surface, promoting microbial clearance and infection risk reduction. This is particularly applicable to acute wounds or contaminated high-risk wounds in which early immune stimulation can dramatically influence healing outcomes.

In our study, the upregulation of NO production concurs with the pro-inflammatory action of these compounds, suggesting that Citrus honey can possess a natural immunomodulatory capacity. This synergy of immune activation and early migratory support locates Citrus honey as a valuable agent to move from the inflammatory phase into the

initiation of the proliferative phase. Yet the elevated pro-inflammatory profile of Citrus honey warrants careful usage in chronic wounds or already inflamed wounds.

As for the keratinocytes results, the absence of cytotoxicity preserves keratinocytes from death at antimicrobial concentrations. Simultaneously, Citrus honey's intense NO induction boosts innate microbicidal activity. NO exerts direct microbicide action and has a capacity to augment leukocyte recruitment, and promotes local vasodilation, facilitating immune cell entry and nutrient provision to the wound bed.

Upon combination with Citrus honey's species-specific antimicrobial activity, the net result is an early-phase therapeutic effect: efficient killing of vulnerable pathogens without host cell cytotoxicity, together with stimulation of keratinocyte migration to start re-epithelialization. This combination of effects is consistent with short-course, early topical treatment of acutely contaminated wounds with Citrus honey, but warns against persistence with chronically inflamed lesions.

Chronic wounds often have a chronic inflammatory process with an abundance of pro-inflammatory cytokines, proteases, and ROS that together prevent transition to the proliferative phase (Eming, Martin, & Tomic-Canic, 2014). In such conditions, the further induction of inflammatory mediators by Citrus honey could potentially initiate further tissue loss and not be healing-promoting. Therefore, while Citrus honey appears to be valuable for acute, infected, or immunocompromised wounds, its application in chronic wounds may have to be closely followed or supplemented with agents that can modulate or inhibit inflammation in later stages.

5.5.3 Alfalfa Honey: moderate antimicrobial effects and height Equilibrium Proliferation

Alfalfa honey had a broad low IC_{50} in batch assays but intermediate MICs at the species level. This is most typical of a generalist antibacterial mechanism (assumed to be H_2O_2

production, osmotic/acidity effects, and a moderate phenolic load), reducing overall bioburden across a range of taxa without reaching very low, species-specific MICs.

Clinically and biologically, this effect is permissive of Alfalfa's strong pro-proliferative and pro-migratory activities: by diminishing the total microbial burden to subcritical levels, Alfalfa honey can remove the major inhibitory burden that keeps keratinocytes from entering the proliferative program, and its mild NO induction avoids chronic inflammation, which otherwise would hinder repair.

In addition, Alfalfa honey was found to have bioactive phenolic acids and flavonoids like caffeic acid and apigenin, and it is also associated with mitogenic and anti-apoptotic signaling of skin cells. These substances may act synergistically to activate growth-related pathways like extracellular signal-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/Akt cascades, which both play a role in the proliferation and survival of keratinocytes (Diegelmann & Evans, 2004) .

Alfalfa honey also displayed long-term facilitation of keratinocyte migration. Facilitated migration following Alfalfa honey treatment could be a result of its ability to control the microenvironment of the wound. Phenolics can also regulate matrix metalloproteinases (MMPs), enzymes that play a dual role of degrading broken tissue while allowing new tissue deposition. By regulating MMP activity, Alfalfa honey would most likely facilitate effective keratinocyte migration with minimal tissue damage, an occurrence replicated by this study's relative wound closure effectiveness.

The other significant finding of this study was the moderate induction, by Alfalfa honey, of nitric oxide (NO), a component immune response and tissue healing mediator. NO performs a two-faced role during wound healing: in early phases, it acts as a signaling molecule to recruit immune cells, combat infection, and induce angiogenesis; in later phases, its concentration needs to decline in order to enable progression towards proliferation and remodeling (Gurtner, Werner, Barrandon, & Longaker, 2008).

Chemical analysis of Alfalfa honey indicates that it is particularly suited to the process of transitioning from inflammation to proliferation, a process often disrupted in pathologic wounds. In acute wounds, where rapid closure is critical to avoiding infection, Alfalfa honey's

stimulation of keratinocyte function can significantly shorten the time of healing. This is especially important in surgical wounds, where early re-epithelialization will minimize dehiscence and infection risks.

In diabetic and vascular insufficiency patients with chronic wounds, the foremost obstacle is that they are unable to progress beyond the inflammatory phase due to oxidative stress, bacterial invasion, and dysregulation of cellular responses. Alfalfa honey's partial induction of NO and its likely antioxidant action will help to foster this concordance by suppressing uncontrolled inflammation and restoring keratinocyte proliferation and migration. In the case of heavily infected wounds proven to contain highly pathogenic organisms, Alfalfa may be best utilized in conjunction with more targeted antimicrobial therapy or after a limited, species-oriented debridement/antimicrobial course.

5.5.4 Avocado Honey: potent antimicrobial activity, height Anti-Inflammatory Proliferative Agent for Chronic Wounds

Avocado honey had a low IC_{50} in all bacteriological tests and very effective, bactericidal action against Bacillus subtilis Such a dose–response curve is characteristic for mechanisms that cause irreversible damage to bacteria such as high rates of H_2O_2 production in assay conditions, close flavan-3-ols and other phenolics (epicatechin, kaempferol, protocatechuic acid) accumulation that remain localized at Gram-positive envelopes, and possible small peptides or reactive compounds that disrupt cell-wall biosynthesis, autolysin activation or spore germination pathways..

Importantly, these direct bacteriostatic actions augment avocado's host-directed profile — potent keratinocyte proliferation and migration at low concentrations with blockade of LPS-induced NO. The result is a complementary therapeutic window: at optimal low concentrations, avocado honey is pro-re-epithelialization and its bactericidal moiety reduces Gram-positive burden and biofilms that are typically responsible for perpetuation of chronic wounds. However, the biphasic mode of action of avocado's cellular response (stimulatory at low doses and inhibitory at high doses) implies caution to achieve a balance between antimicrobial efficacy and safe host exposure.

As a bonus to proliferation, avocado honey had the strongest pro-migratory activity among the honeys analyzed by virtue of increased wound closure in the scratch assay. Migration of keratinocytes into the wound bed is a condition for epithelialization, and slowing this can result in prolonged exposure to environmental stress, infection, and consequent tissue injury. In our study, the monolayers of keratinocytes treated with avocado honey closed nearly all by 48 hours, higher than citrus, alfalfa, and even the control Manuka honey values.

Avocado honey also significantly inhibited this NO overproduction with disease, indicating an acute anti-inflammatory effect. This can be attributed to its high antioxidant content of polyphenols, whose role is to scavenge reactive oxygen and nitrogen species, as well as to its ability to modulate key inflammation-related pathways such as NF-κB and iNOS expression. The dual advantage of reduction in oxidative burden and direct downregulation of pro-inflammatory mediators puts avocado honey as a useful adjunct to excess inflammation control but without preventing the initial, desired inflammatory response required for debridement and pathogen clearance.

The combination of proliferative, migratory, and anti-inflammatory and antimicrobial action, the honey is optimally suited for chronic infected ulcers in which infection management and restoration of proliferative potential are required, which means that Avocado honey will be effective in the treatment of multi-factorial pathology of chronic wounds. Unlike acute wounds, where the inflammation resolves in a normal manner, chronic wounds are trapped in a low-grade but persistent state of inflammation that interferes with cellular processes required for healing.

5.5.5 Manuka Honey: Potent yet Dosage-Dependent Wound Healing Agent

Manuka honey has been rated as among the most potent natural wound healing agents because of its unique phytochemical content, with high levels of methylglyoxal (MGO), phenols, and hydrogen peroxide-producing capacity.

However, in this study, evidence of a distinctly biphasic Manuka honey biological effect shows the need for individually optimal dose adjustment in wound care treatments aimed at cure. Manuka honey increased keratinocyte proliferation and migration at low concentrations, which plays a huge role in re-epithelialization during wound healing's proliferative phase. Conversely, at high concentrations, it showed highly severe cytotoxicity, not just to cell survival, but even to the migratory capacity of keratinocytes—critical to wound repair. This bipolarity reveals the very thin line between therapeutic action and cytotoxicity, particularly in wound therapy.

This concurs with previous research that Manuka honey triggers cytoskeletal reorganization, triggers ECM deposition, and increases integrins and MMPs that are vital for cell (Ranzato , Martinotti, & Burlando, 2012) .

Significantly, low-dose Manuka honey also helps in antimicrobial protection without host cytotoxicity. Its natural non-peroxide action, mainly MGO-mediated, helps in the inhibition of a wide range of wound bacteria, including Staphylococcus aureus and Pseudomonas aeruginosa, and reduces the risk of infection and enables unbroken tissue healing (Jenkins, Cooper, & Burton, 2011). The ability to protect against infection and to stimulate keratinocyte function at appropriate concentrations makes Manuka honey a valuable adjunct to the healing of slow- or chronic-healing wounds.

Compared to the above positive effects, Manuka honey with high doses in this study considerably suppressed keratinocyte viability and disrupted their migratory ability. It is highly likely because of its high concentration of MGO. While MGO is accountable for Manuka's broad spectrum of antimicrobial activity, toxic concentrations are cytotoxic to mammalian cells by its powerful protein- and DNA-modifying activities, resulting in advanced glycation end products (AGEs), oxidative stress, and mitochondrial damage (Afrin, et al., 2018).

These cytotoxic cell activities could retard the proliferative phase of wound healing by preventing effective keratinocyte colonization of the wound surface and preventing granulation tissue formation.

Secondly, high concentrations of Manuka honey can interfere with the ideal redox state required for the healing of wounds. Although low levels of ROS act as signaling molecules

to enhance healing, excessive levels of ROS can induce apoptosis, inhibit angiogenesis, and interfere with ECM remodeling (Diegelmann & Evans, 2004) .This is consistent with our observation of inhibited migration in scratch assays at high concentrations, which implies that highly concentrated Manuka honey may paradoxically inhibit the very processes it is designed to activate.

These results have significant clinical relevance when considering the use of Manuka honey. Although its antimicrobial action renders it a potential wound therapeutic agent, therapeutic effectiveness relies on the achievement of an optimal balance of its bioactive components to avoid cytotoxicity.

Dosage regimens might be customized with specific concentrations depending on wound class and phase, such as reducing concentrations in the proliferative phase to maximize cell proliferation but enhancing slightly at the end of the inflammatory phase for infection control without unnecessary exposure. Moreover, the incorporation of Manuka honey with other dressings (e.g., nanofiber scaffolds, hydrogels) might reduce its cytotoxicity while ensuring antimicrobial activity (Maddocks & Jenkins, 2013).

5.6 Honey color interruption with the results

An interesting secondary result from this study was the perceived influence of honey color on biological activity. Lighter honeys were likely to induce extreme pro-inflammatory reactions in THP-1 cells and have reduced proliferative potential in HaCaT keratinocytes, whereas darker honeys were found to induce higher rates of cell proliferation. These findings suggest that the color of honey can, in turn, serve as a key to concealed differences in bioactive content, i.e., phenolic compounds and antioxidant capacity.

Although honey color was not a key factor in this study, the reproducibility of these results in multiple assays indicates that it could be an initial biomarker for biological activity. Further investigation into the interaction among honey color, chemical composition, and functional effect is needed to further define this proposed relationship and its implications

for selecting honey varieties for use in wound healing treatments (Becerril-Sánchez, Quintero-Salazar, Dublán-García, & Escalona-Buendía, 2021).

5.7 Study Strengths

The study combines tightly controlled laboratory experiments with complementary approaches in order to give a global, mechanistic picture of how different monofloral honeys both interact with host cells and microbes. Simultaneous screening of all honeys side-by-side under the same laboratory conditions minimized inter-assay variability and allowed for direct, head-to-head comparison, which is not common in the literature.

Additionally, a wide range of biologically pertinent endpoints (MTT-based keratinocyte proliferation/viability, scratch assay-based collective cell migration, inflammatory modulation by Griess, antimicrobial MIC/MBC/IC_{50} assessment, and molecular docking) was used, pooled together to generate phenomenological data alongside mechanistic hypotheses. The use of well-characterized cell models (HaCaT keratinocytes and LPS-stimulated THP-1 macrophage-like cells) means that results translate directly to a broad literature on wound-healing.

The inclusion of a medical-grade Manuka control offers a translational context and comparators to benchmarks. Finally, the study was multidisciplinary (cell biology, microbiology, analytical chemistry, in-silico modeling), which adds plausibility and interpretability of mechanistic links between composition and bioactivity.

- Side-by-side controlled comparison under the same experimental conditions.
- Multi-endpoint design (cellular assays + antimicrobial testing + in silico docking) for mechanistic scope.
- Employment of well-established, widely accepted wound-relevant cell models (HaCaT, THP-1).
- Incorporation of a clinical standard (medical-grade Manuka).
- Translational focus: dosing, formulation considerations, and clinically relevant microbes considered.

5.8 Limitations and Caveats

While the research provides informative mechanistic and comparative data, some key limitations restrict direct clinical extrapolation and set priorities for future research. All experiments were performed in vitro under static culture conditions that do not reflect the full complexity of real wounds (interacting immune cell subsets, blood supply, angiogenesis, innervation, dynamic exudate, protease, or full biofilm ecology). The Griess assay measures nitrite in supernatants and is an indirect indicator for NO production—interpretation of inflammatory modulation would be complemented by direct iNOS mRNA/protein assays and a broader cytokine panel.

In silico docking gives testable hypotheses for target interactions but cannot substitute for biochemical binding or functional enzyme inhibition measurements, and also does not account for uptake, metabolism, or protein conformational mobility within cells.

Antimicrobial MIC/MBCs are valuable but obtained under artificial broth conditions; they fail to reflect activity in wound exudate, biofilm matrices, or when honey is incorporated into a dressing. Finally, honey is a variable natural commodity—a single-batch sample, and the restricted number of floral sources utilized tested cannot reflect the complete seasonal, geographic, or processing range found in commercial honey. The overall caveats and how they might affect interpretation:

- Sample / replicate limitations and statistical power: there were few independent biological replicates (see Methods). Small effect sizes and subtle interactions may consequently be underpowered and require validation in larger sample sets.
- Limited microbial panel: the antibacterial testing used a limited panel of organisms; broader pathogen panels (e.g., MRSA, Pseudomonas biofilm, mixed-species wound microbial consortia) would better define clinical utility.
- Time course and concentration range: assays focused mainly on initial (24–48 h) endpoints and a limited grid of concentrations. Longer time courses, more precise

dose–response curves and kinetics would better define therapeutic windows and chronic exposure risks.

- Cell models: HaCaT and THP-1 are easily accessible, well-characterized lines, but are monoculture and immortalized systems. Primary human keratinocytes, primary macrophages, fibroblasts, and co-culture models (keratinocyte–fibroblast, neutrophil–macrophage, or 3D skin equivalents) would be more physiologically relevant.
- Docking limitations: predicted affinities do not take into account bioavailability, transport within cells, enzymatic metabolism of phenolics, or matrix sequestration by proteins and sugars. Docking needs to be complemented by enzyme inhibition assays and cell-based target engagement experiments.
- Safety and systemic impacts: in vitro cytotoxicity is not always related to local tolerance, allergic hazard, or systemic uptake in man—initial clinical and comparative animal safety experiments are warranted before therapeutic counsel.

5.9 Recommendations For Future Work

Although the above study compared monofloral honeys comprehensively using wound-healing–relevant in vitro models, several avenues are still open for further research. First, extending this work into in vivo wound models may allow for the cellular and antimicrobial effects observed to be validated in physiological conditions i. Second, dose-optimization studies using controlled-release formulations, such as hydrogels, nanofiber scaffolds, or films, are needed for defining clinically relevant concentration windows, especially in honeys showing biphasic behavior like Manuka and Avocado honey.

Further studies should also involve inflammatory profiling in a time-resolved manner, looking into cytokine expression, macrophage polarization markers, and markers of oxidative stress across phases of wound healing. Chemical characterizations through LC-MS/MS combined with metabolomic approaches would enable the identification of active principles

responsible for the observed bioactivities. Lastly, the combination of selected honeys with standard wound care agents or antibiotics might give a clear perspective on their use as adjunct therapies, especially for chronic or infected wounds.

5.10 Conclusion Of The Discussion

In conclusion, it is clear from this research that various monofloral honey types exhibit disparate effects on keratinocyte proliferation and migration, which represent central aspects of wound healing. Avocado and Alfalfa honeys act as adjuncts to keratinocytes to induce re-epithelialization, thereby serving as useful remedies for inflamed or chronic wounds. Citrus or Manuka honeys, on the other hand, act as useful remedies for acute wounds.

This highlights the importance of applying the appropriate phase of honey to wounds. However, the study is considered to lack relevance since it is done in vitro. Additionally, the study only involves a few types of honey; therefore, the results might not accurately reflect the condition of wounds within the human body. Future studies should involve ex vivo research.

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التقييم المختبري لاثار التئام الجروح لثلاثة أنواع من العسل الفلسطيني باستخدام خلايا من خط الخلايا الكيراتينية البشرية

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الملخص باللغة العربية

يُعد التئام الجروح عملية بيولوجية معقدة تتطلب تنسيقًا دقيقًا بين أنشطة خلوية متعددة، مثل تكاثر الخلايا الكيراتينية وهجرتها، وتنظيم الالتهاب، والحماية المضادة للميكروبات. وقد استُخدم العسل تقليديًا في علاج الجروح، إلا أن البيانات العلمية المتاحة التي تقارن الأنشطة البيولوجية لأنواع العسل المختلفة لا تزال محدودة. في هذه الدراسة، تم فحص استخدام ثلاثة أنواع من العسل الفلسطيني، وهي: عسل الأفوكادو، وعسل البرسيم الحجازي، وعسل الحمضيات.

استُخدمت خلايا HaCaT لتقييم حيوية الخلايا وتكاثرها وقدرتها على الهجرة وإعادة الظهارة باستخدام اختبار الخدش، في حين تم اختبار الاستجابة الالتهابية في خلايا THP-1 من خلال قياس إنتاج أكسيد النيتريك باستخدام اختبار غريس. إضافة إلى ذلك، جرى تحليل الخصائص المضادة للبكتيريا لكل نوع من العسل ضد مسببات الأمراض الموجودة في الجروح. كما أُجريت دراسات الالتحام الجزيئي لتحديد التفاعلات المحتملة بين المركبات الحيوية الموجودة في العسل والأهداف الجزيئية الرئيسية المشاركة في التئام الجروح.

أظهرت النتائج بوضوح أن جميع عينات العسل كان لها تأثيرات إيجابية ومميزة على عمليات التئام الجروح. وقد فاق تعزيز انغلاق الجرح وهجرة الخلايا الناتج عن عسل الأفوكادو وعسل البرسيم الحجازي تأثير أنواع أخرى من العسل مثل عسل الحمضيات، مما يدل على دور أكبر لهذه العينات في عملية إعادة الظهارة أثناء التئام الجروح. كما أظهرت عينات العسل مستويات من الخصائص المضادة للالتهاب والمضادة للبكتيريا، مما يشير إلى أن اختلاف مستويات المركبات الكيميائية هو العامل الأساسي في تباين أداء عينات العسل. وأكدت دراسة الالتحام الجزيئي ذلك من خلال إظهار تفاعلات إيجابية بين مركبات العسل والبروتينات المشاركة في عمليات مثل هجرة الخلايا ومسارات الالتهاب.

تسلط هذه الدراسة الضوء على إمكانات العسل الفلسطيني كبديل أو مكمل للعلاجات الحديثة للجروح، كما تؤكد فعاليته من خلال دعم استخدامه التقليدي طويل الأمد بدراسة مخبرية.

الكلمات المفتاحية: التئام الجروح، أنواع العسل الفلسطيني، الخلايا الكيراتينية، الاستجابة الالتهابية، عملية إعادة الظهارة