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THE BIOACTIVITY OF PROTEINS IN SOUTHERN AFRICAN FYNBOS HONEY

by

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Abstract

Medical grade honey aids in wound debridement, reducing inflammation and infection, and promoting cellular infiltration due to its sugar content, acidic pH, glyoxal, hydrogen peroxide, polyphenols, and peptides. The contribution of the protein fraction of honey to these activities is not well understood. Investigating the bioactivity of proteins isolated from honey could lead to the discovery of novel antimicrobial, anti-inflammatory, and wound-healing agents with potential applications in medicine, functional foods, and pharmaceuticals. This research fills critical knowledge gaps by characterizing honey proteins, elucidating their mechanisms of action, and assessing their stability, bioavailability, and therapeutic potential, offering new insights beyond the well-studied carbohydrate and polyphenol components of honey. This study evaluated the bioactivity of whole honey and corresponding isolated protein fractions from Southern African Fynbos (FB) honeys which were compared to the medically approved Manuka (MA) honey and a Buckwheat (BU) honey as controls.

The protein fractions of five FB honeys and the control honeys were isolated using gel filtration chromatography. Analyses included protein content, total polyphenol content (TPC), sugar content, and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. At tested non-cytotoxic concentrations, the effect of the whole honey and the protein fractions on cellular migration was evaluated with the scratch assay using the HaCaT keratinocyte and the SC-1 fibroblast cell lines. Antioxidant activity was evaluated with the *in vitro* oxygen radical absorbance capacity (ORAC) assay and the Trolox equivalent antioxidant capacity (TEAC) assay. The cellular antioxidant activity (CAA) was also determined using HaCaT and SC-1 cell lines. Nitric oxide (NO) mediated effects on inflammation were evaluated in the *in vitro* lipopolysaccharide (LPS) induced NO scavenging/RAW 264.7 cell model. Antibacterial activity in terms of minimum inhibitory concentrations (MIC's) against *Escherichia coli* (*E. coli* ATCC 700928) and *Staphylococcus aureus* (*S. aureus* DSM 2569) were determined.

The protein content varied across honey samples, with BU (1.33 ± 0.15 mg/g) and FB6 (1.19 ± 0.1 mg/g) showing significantly higher levels than MA (0.95 ± 0.07 mg/g, $p < 0.0001$), while FB2 (0.78 ± 0.08 mg/g) and FB3 (0.72 ± 0.06 mg/g) had significantly lower levels ($p < 0.0001$). The protein fractions showed a similar trend, with MF1 (0.05 ± 0.01 mg/g), FB1 (0.05 ± 0.01 mg/g), FB2 (0.07 ± 0.02 mg/g), and FB3 (0.04 ± 0.01 mg/g) exhibiting significantly lower concentrations than MA (0.12 ± 0.01 mg/g, $p < 0.0001$).The sugar/fructose content in honey ranged from 25.18 ± 0.10 mg/100 g (MF1) to 38.99 ± 0.18 mg/100 g, with MF1 showing significantly lower values than MA ($p = 0.0212$). In protein fractions, sugar content was highest in MA (15.95 ± 3.15 mg/100 g) and lowest in BU (11.07 ± 0.63 mg/100 g). Total phenolic content (TPC) was highest in BU (1.05 ± 0.03 mg GAE/g, $p = 0.0273$) and lowest in MF1 (0.47

± 0.04 mg GAE/g, p = 0.0002), with FB2 and FB3 also significantly lower than MA (p < 0.0001). Molecular mass analysis confirmed the presence of distinct protein bands between 45-70 kDa.

Cell viability in HaCaT and SC-1 cells exceeded 80% at tested concentrations. Wound closure rates in HaCaT cells for whole honey (1.25% v/v) ranged from 71 ± 8% to 90 ± 3%, and for protein fractions (0.0044-0.0146 mg/g) from 75 ± 8% to 89 ± 3%. In SC-1 cells (0.625% v/v), rates ranged from 46 ± 15% to 85 ± 3% for whole honey and 66 ± 11% to 86 ± 6% for protein fractions.

Antioxidant capacity (TEAC) was highest in BU honey (8.66 ± 0.14 µM TE/g) and lowest in FB2 (2.05 ± 0.35 µM TE/g). ORAC values ranged from 1.55 ± 0.40 to 2.67 ± 0.73 µM TE/g. Protein fractions showed minimal antioxidant activity. Oxidative damage in HaCaT cells ranged from 82.2 ± 4.4% to 100.0 ± 8.2% (honey) and 107.9 ± 5.3% to 117.1 ± 7.3% (protein). In SC-1 cells, damage was 79.1 ± 3.1% to 95.1 ± 5.6% (honey) and 87.8 ± 4.1% to 95.0 ± 8.5% (protein). NO levels were similar across samples. Anti-inflammatory activity was highest in BU honey (1.60 ± 0.25 µM), while FB9 (8.78 ± 2.90 µM) which had significantly lower activity than MA (p = 0.0353) and BU (p = 0.0002). Protein fractions exhibited lower NO scavenging, particularly BU (10.45 ± 0.47 µM, p = 0.0265).

Antibacterial activity (MIC50) against *E. coli* was strongest in MF1 (26.8 ± 6.4 mg/mL) and BU (29.9 ± 6.4 mg/mL). Against S. aureus, MF1 (0.01 ± 0.01 mg/mL) and FB1 (0.05 ± 0.04 mg/mL) had the highest efficacy among protein fractions, while BU and MF1 honeys showed no activity.

Tentatively, the proteins in the honey samples and protein fractions could be assigned to the major royal jelly protein (MRJP) family, glucose oxidase and bee defensin-1 (DF-1) in the based on molecular mass. The protein fraction did not contribute significantly to the antioxidant activity of honey nor to the NO scavenging activity *in vitro*. For several honeys and protein fractions the MIC_{50} was determined and generally, the honey and protein fractions showed increased targeting of *E. coli* than *S. aureus* similar to Manuka honey. FB honeys exhibited anti-inflammatory, antioxidant, and antimicrobial properties similar to the MA honey control. The findings highlight the potential of Southern African Fynbos honeys to exhibit bioactivities comparable to medically approved honeys, suggesting their promising role in wound care and antimicrobial applications.

Keywords: Fynbos, Manuka, Buckwheat, honey proteins, antioxidant, antibacterial, immune modulatory, cellular migration.

Research Outputs

Conferences:

Podium presentation at the 56th annual conference of the South African Society for Basic and Clinical Pharmacology (SASBCP) held on the 24th-27th of August 2023 at Rhodes University.

Podium presentation at the 57th annual conference of the SASBCP held on the 15th-17th of September 2024 at Irene conference centre.

List of Abbreviations, Symbols, Chemical Formulae

Abbreviation	Full term
α	Alpha
β	Beta
δ	Delta
ε	Epsilon
γ	Gamma
%	Percentage
AU	Absorbance units
µ	Micro
kDa	Kilodalton
pH	Negative logarithm of H^+ activity
°C	Degrees Celsius
3D	Three dimensional
A	
AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid
ACN	Acetonitrile
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
B	
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
C	
CAA	Cellular antioxidant activity
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
CV	Crystal violet
D	
DCFH-DA	2' 7' dichlorodihydrofluorescein diacetate
ddH_2O	Deionised water
DF-1	Bee-defensin-1
E	
ECM	Extracellular matrix

EGF	Epidermal growth factor
F	
FCS	Fetal calf serum
FOX-1	Ferrous iron oxidation xylenol orange
G	
GAE	Gallic acid equivalents
GO	Glyoxal
GOx	Glucose oxidase
GP	Glycoprotein
H	
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric acid
H_2SO_4	Sulfuric acid
I	
IEF	Isoelectric focusing
IL	Interleukin
iNOS	Inducible nitric oxide synthase
K	
$K_2S_2O_8$	Potassium persulphate
L	
LC-MS/MS	Liquid chromatography–mass spectrometry
LPS	Lipopolysaccharide
M	
MDR	Multidrug-resistant
MGO	Methylglyoxal
M-H	Mueller-Hinton
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase
MRJP	Major royal jelly protein
MRP	Maillard reaction products
MRSA	Methicillin resistant *Staphylococcus aureus*
N	
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NaO_2	Sodium superoxide
NADPH	Nicotinamide adenine dinucleotide phosphate

NED	N-1-naphthylethylenediamine
NET	Neutrophil extracellular traps
NHS	National Health Service
NO	Nitric oxide
O	
OH	Hydroxide
ORAC	Oxygen radical absorbance capacity
OprF	Outer membrane protein F
P	
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
pI	Isoelectric point
u-PA	Urokinase-type plasminogen activator
u-PAR	Urokinase-type plasminogen activator receptor
R	
Rf	Retention factor
ROS	Reactive oxygen species
S	
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEM	Standard error of mean
SNP	Sodium nitroprusside
SPE	Solid phase extraction
SRB	Sulforhodamine B
T	
TEAC	Trolox equivalent antioxidant capacity
TGF	Transforming growth factor
TLR	Toll like receptor
TNF	Tumour necrosis factor
TPC	Total polyphenol content
V	
VEGF	Vascular endothelial derived growth factor
vWF	von Willebrand factor

List of Figures

Figure 2.2.1. The wound healing cascade phases and appropriate duration of healing in (a) acute wounds and (b) chronic wounds. 6

Figure 2.2.2. A schematic diagram of the initial haemostasis phase within wound healing as an immediate response at the wound site depicting the platelet plug and growth factors such as platelet derived growth factor (PDGF).....9

Figure 2.2.3. A schematic diagram of the inflammatory phase of wound healing depicting several cellular role players such as neutrophils, mast cells, and macrophages.....10

Figure 2.2.4. A schematic diagram of (a) cell migration and the subsequent tissue remodelling involving collagen deposition with an observed reduction of inflammatory processes and completed angiogenesis. 12

Figure 2.4.1. The hypothesised mechanism by which Manuka honey inhibits (a) *S. aureus* (MRSA) and (b) *P. aeruginosa*.....20

Figure 2.6.1. The fundamental activity of sugars, H_2O_2, and various enzymes found within honey. 24

Figure 2.7.1. The chemical structure of the (a) phenolic acids and (b) flavonoids commonly found within honey. 27

Figure 2.8.1. Scanning electron micrograph of GP-induced morphological changes and subsequent cell death of *E. coli* and *B. subtilis* in lag phase of the growth cycle. 30

Figure 3.4.1. The detection of protein and sugar content in sample FB9, extraction 1, using (a) the Coomassie blue spot test and (b) the Seliwanoffs spot test, respectively, in the fractions following separation using gel filtration chromatography. 46

Figure 3.4.2. The average protein concentration (mg/g) of each 100% honey and the protein fraction. 49

Figure 3.4.3. The average sugar/fructose sugar concentration (mg/g) of each 100% honey and protein fraction.. 50

Figure 3.4.4. Total polyphenol content (mg GAE/g) of the 100% honey and the protein fractions. 51

Figure 3.4.5. The correlation between A_{450} (AU) and TPC (mg GAE/g) of honey samples, the orange line represents the same correlation without BU, the equation of the line, y= 1.4567x + 0.1734, R^2=0.6586, and r= 0.8116.....53

Figure 3.4.6. Representative images of 4-20% SDS-PAGE Tris glycine gels loaded with (a) 15 µg whole honey and (b) 2 µg protein. 53

Figure 4.4.1. The cytotoxicity in HaCat cells of (a) 1.25% (v/v) honey and the (b) 0.0044-0.0146 mg/g protein fraction analysed with the SRB assay.....70

Figure 4.4.2. The cytotoxicity in SC-1 cells of (a) 1.25% and 0.625% (v/v) honey and the (b) 0.0044-0.0146 mg/g protein fraction analysed with the SRB assay 71

Figure 4.4.3. The HaCaT migration induced by 1.25% (v/v) honey and the 0.0044-0.0146 mg/g protein fraction assessed with the scratch assay.....75
Figure 4.4.4. The SC-1 migration induced by 0.625% (v/v) honey and the 0.0044-0.0146 mg/g protein fraction assessed with the scratch assay.....78
Figure 5.4.1. Antioxidant activity, TEAC assay of the honey samples and protein fractions. Data was expressed as Trolox equivalent (TE) µM TE/g.. 92
Figure 5.4.2. Antioxidant activity, ORAC assay of the honey samples and the protein fractions expressed as µM TE/g. 93
Figure 5.4.3. NO levels following the addition of (a) 25% (v/v) honey and the (b) protein fractions expressed as % NO produced by the positive control.....96
Figure 5.4.4. The anti-inflammatory (NO scavenging (+LPS)) in the RAW264.7 cells exposed to (a) 1.25% (v/v) honey and (b) protein fraction.. 99
Figure 5.4.5. The pro-inflammatory (NO scavenging (-LPS)) in the RAW264.7 cells exposed to (a) 1.25% (v/v) honey and (b) protein fraction. Data was expressed as nitrite equivalents (µM) 100
Figure 9.1.1. Replicate 1 of a 4-20% SDS-PAGE Tris Glysine gel indicating the (a) 15 µg whole honey and (b) 2 µg protein and peptide mass profiles of each sample.. 160
Figure 9.1.2. Replicate 2 of a 4-20% SDS-PAGE Tris Glysine gel indicating the (a) 15 µg whole honey and (b) 2 µg protein and peptide mass profiles of each sample. 160
Figure 9.1.3. Replicate 3 of a 4-20% SDS-PAGE Tris Glysine gel indicating the (a) 15 µg whole honey and (b) 2 µg protein and peptide mass profiles of each sample. 161
Figure 9.2.1. The cell migration of 1.25% (v/v) whole honey samples assessed using the scratch migration/HaCaT cell model replicate 1.. 163
Figure 9.2.2. The cell migration of 1.25% (v/v) whole honey samples assessed using the scratch migration/HaCaT cell model replicate 2. 165
Figure 9.2.3. The cell migration of 1.25% (v/v) whole honey samples assessed using the scratch migration/HaCaT cell model replicate 3. 167
Figure 9.2.4. The cell migration of isolated protein fractions assessed using the scratch migration/HaCaT cell model replicate 1. 169
Figure 9.2.5. The cell migration of isolated protein fractions assessed using the scratch migration/HaCaT cell model replicate 2. 171
Figure 9.2.6. The cell migration of isolated protein fractions assessed using the scratch migration/HaCaT cell model replicate 3... 173
Figure 9.2.7. The cell migration of 0.625% (v/v) whole honey samples assessed using the scratch migration/SC-1 cell model replicate 1.....175
Figure 9.2.8. The cell migration of 0.625% (v/v) whole honey samples assessed using the scratch migration/SC-1 cell model replicate 2.....176

Figure 9.2.9. The cell migration of 0.625% (v/v) whole honey samples assessed using the scratch migration/SC-1 cell model replicate 3.....178
Figure 9.2.10. The cell migration of isolated protein fractions assessed using the scratch migration/SC-1 cell model replicate 1. 180
Figure 9.2.11. The cell migration of isolated protein fractions assessed using the scratch migration/SC-1 cell model replicate 2. 182
Figure 9.2.12. The cell migration of isolated protein fractions assessed using the scratch migration/SC-1 cell model replicate 3. 184

List of Tables

Table 2.2.1. Treatment options for chronic and infected wounds healing by secondary intent. 13

Table 2.3.1. The intended use and therapeutic effect of several commercialised honey-based products for wound application 16

Table 2.3.2. A comparison between honey and other bioactive compounds used in the treatment of wounds 18

Table 2.5.1 Physiochemical parameters of Buckwheat and Manuka honeys 22

Table 2.5.2. The proteins including peptides and enzymes identified in several honeys by proteomic studies. 28

Table 2.8.2. The most abundant and molecular mass of proteins found within several honey sources 29

Table 3.4.1. The sample codes, floral type, and area of origin of the honeys used in this study. 45

Table 3.4.2. The repeats, the fractions, and protein and sugar content of each honey sample. 47

Table 3.4.3. Physiochemical properties of the honey samples and the relative amounts of fructose, and polyphenols in the protein fractions. 52

Table 3.4.4. Protein bands detected in the honey samples analysed using SDS-PAGE, the protein bands and retention factor (Rf) were identified using ImageLab (BioRad, Lasec, Western Cape, SA) 55

Table 4.4.1. The HaCaT and SC-1, wound closure (%) of honey and the protein fraction evaluated with the scratch assay. 80

Table 5.4.1. Pro-oxidant and antioxidant (% oxidative damage (%OD)) evaluated in HaCaT and SC-1 cells exposed to honey (% (v/v)) at 1.25% and 0.625%, respectively and the protein fractions analysed with the DCFH-DA assay. 94

Table 5.4.2. The percentage density of the RAW 264.7 cells after the anti- or pro-inflammatory studies evaluated with the Crystal Violet assay. 101

Table 5.4.3. Pearson coefficient (r), correlation coefficient (r^2), p values and significance* of honey and the protein fractions for TPC, the antioxidant and the anti-inflammatory assays. 102

Table 6.4.1. The MIC_{50} of the honey and protein fractions against *E. coli* (ATCC 700928) and *S. aureus* (DSM 2569). 118

Declaration

I declare that the dissertation, which I hereby submit for the degree MSc Anatomy at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Ethics Statement

The author, whose name appears on the title page of this dissertation, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

Signature
Chloé Jade Stragier
2024

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Table of Contents

Chapter 1: Introduction....1

Chapter 2: Literature review....3

2.1 Introduction....3

2.2 Wounds....3

2.2.1 Wound healing mechanisms....3

2.2.2 Acute vs chronic wounds....4

2.2.3 Wound healing phases....8

2.2.4 Treatment of chronic wounds....13

2.3 Medicinal honey....16

2.4 Manuka honey....19

2.5 Buckwheat honey....21

2.6 Honey constituents....22

2.7 Honey polyphenols and associated anti-inflammatory and antioxidant activity....25

2.8 Honey protein and associated antimicrobial activity....28

2.9 South African honey research....32

2.10 Apicultural influence and potential limitations of honey....32

2.11 Project rationale....34

2.12 Aims....35

2.13 Objectives....36

Chapter 3: The protein isolation and physiochemical properties of selected South African honeys and protein extracts....37

3.1 Introduction....37

3.2 Materials....38

3.3 Methods....40

3.3.1 Protein isolation from whole honey samples....40

3.3.2 Determination of physiochemical properties....42

3.4 Results....45

3.4.1 Honey samples....45

3.4.2 Protein isolation....46

3.4.3 Physiochemical properties 48
3.4.4 Molecular mass profile determination 53
3.5 Discussion 57
3.5.1 Honey adulteration in a South African context 57
3.5.2 Physiochemical determination 57
3.5.3. Protein determination and tentative identification 59
3.6 Conclusion 62
Chapter 4: The cytotoxicity and cell migratory activity of selected South Africa Fynbos honeys and protein extracts 63
4.1 Introduction 63
4.2 Materials 65
4.3 Methods 65
4.4 Results 69
4.4.1 Cytotoxicity 69
4.4.2 Cell migration 72
4.5 Discussion 81
4.6 Conclusion 84
Chapter 5: The antioxidant and anti-inflammatory activity of selected South African honeys and protein extracts 85
5.1 Introduction 85
5.2 Materials 87
5.3 Methods 88
5.4 Results 92
5.4.1 Antioxidant activity 92
5.4.1 Inflammatory activity 95
5.5 Discussion 103
5.6 Conclusion 111
Chapter 6: The antibacterial activity of selected South African honeys and protein extracts 113
6.1 Introduction 113

6.2 Materials....114
6.3 Methods....115
6.4 Results....116
6.5 Discussion....119
6.6 Conclusion....122
Chapter 7: Concluding discussion....124
7.1 Summary of results....124
7.2 Limitations and recommendations....128
Chapter 8: References....132
Chapter 9: Appendices....160
9.1 Appendix A: Chapter 3 supplementary data....160
9.2 Appendix B: Chapter 4 supplementary data....162
9.3 Appendix C: Ethics approval....185

Chapter 1: Introduction

It is evident from archaeological findings that honey was used by the Assyrians, Chinese, Egyptian, Greek, and Roman civilizations[1,2], all of whom consumed honey for nutritional benefits and medicinal properties.[3] Archaeological evidence came in the form of Ancient scrolls, Sumerian clay tablets (6200 BC), Egyptian papyri (1900–1250 BC), Hindu scripture (5000 years), the Quran (Islamic holy book), the Bible (Christian holy book), and Hippocrates (460–357 BC).[3,4] A Greek scientist, Hippocrates, described medicinal honey mixtures for baldness, contraception, laxative action, topical antiseptic, treatment of scars, eye diseases, and acute fevers.2 Honey is described as a healthy drink in Islamic medicine, considered as a treatment of diarrhoea and tuberculosis.[2] Transliterated from hieroglyphic symbols the Egyptian civilisations used honey to embalm their dead and heal infected wounds due to documented antibacterial properties.[2] Beyond historical reverence, honey has been recognized in traditional medicine for treating digestive issues, skin disorders, and infections.

More recent research has identified honey's ability to debride wounds;[4] reduce chronic inflammation; decrease wound pH; and promote infiltration of fibroblasts at the wound site.[1] With this, modern researchers have rediscovered honey's ability to treat burns, infected and chronic wounds, ulcers, and boils.[5] Additionally, its ability to disrupt bacterial biofilms has led to the development of honey-based wound dressings, with clinical studies reporting improved wound healing and reduced scarring.[4,6] Therefore, modern research has reaffirmed honey's medicinal potential, particularly in wound healing. This medicinal potential has been attributed to the bioactivity of honey, which includes the antimicrobial, anti-inflammatory, and antioxidant properties. These effects are largely due to its biochemical composition, particularly its sugar content, with fructose (~38%) and glucose (~31%) being the primary components,[2] vitamins, minerals, enzymes, flavonoids, and phenolic acids.[4]

Despite extensive research on honey's abovementioned biochemical components and associated bioactivity, limited studies have explored the therapeutic properties of proteins and peptides isolated from honey, particularly in honey from South Africa. For example, a study done by Erban *et. al.*[7] analysed the protein content within several European honey's, identifying and characterising the most abundant proteins.[7] However, the associated bioactivity of the proteins identified was not analysed. This research seeks to contribute to the growing understanding of honey's medicinal value while highlighting the unexplored potential of its protein components. By researching the protein component of honey critical knowledge gaps are addressed such as characterising honey proteins, elucidating their mechanisms of

action, and assessing their stability, bioavailability, and therapeutic potential, offering new insights beyond the well-studied carbohydrate and polyphenol components of honey.

South African honey is of interest in this study due to the unique Fynbos region, a hyper diverse biosphere consisting of ±7000 plant species acting as a unique source of South African honey.[8] This region is pollinated by, the Cape honeybee, *Apis mellifera capensis*, unique to the Western Cape and some regions of the Eastern Cape and differs from the native honeybee, *Apis mellifera scutellate,* predominantly found in the central and southern regions of Africa.[9] Cape honeybees are primary consumers of the Fynbos biome therefore pollinating the region assisting in reproduction of flowing plants.[9] Given the ecological uniqueness of Fynbos honey, investigating its protein content and associated bioactivity could provide novel insights into its potential therapeutic applications. Thus, this study's purpose is to bridge the gap in wound healing research by exploring the associated bioactivity of the proteins found in Southern African honey.

Chapter 2: Literature review

2.1 Introduction

Honey is a natural product made by honeybees that forage the floral nectar, plant secretions, and/or excretions of insects from various plant species. It is regarded as a nutritious, healthy and natural food product whose composition is dependent on its botanical and geographical origins. Consequently, honey is categorised by its botanical origin into two types: (a) monofloral or (b) multifloral. Monofloral honey is derived from the nectar or honeydew of a single predominant botanical species, whereas multifloral honeys are produced from several botanical species.[10]

The health benefits of honey is primarily attributed to the acidity, high osmolarity, and ability to produce hydrogen peroxide (H_2O_2) and nitric oxide (NO) upon contact with water. Moreover, honey contains non-peroxide factors such as methylglyoxal (MGO), which collectively contribute to the antimicrobial activity. In addition to these components, honey is rich in phenolic compounds, organic acids, enzymes, and nutrients all of which exhibit potential anticancer, antiparasitic, antidiabetic and anti-inflammatory bioactivity.[11]

2.2 Wounds

A wound is defined as any break in the continuity of the skin, also described as the disruption of normal anatomical structures and functions. Wounds have remained a consistent clinical challenge in pathology throughout history.[12] The earliest medical manuscript, a clay tablet (2200 BC), described the three healing gestures: washing the wound, making the plasters, and bandaging the wound.[13] Hippocrates (377-460 BC) was a Greek physician and surgeon who used vinegar (acetic acid) to wash open wounds and bandaged wounds in dressings to prevent further injury. Galen, a Roman surgeon, was the first to note that pus from wounds, inflicted by the gladiators, preceded wound healing.[14] In the 1800's, Joseph Lister recognized that anti-sepsis could prevent infection by placing carbolic acid on fractures to sterilize the wound, subsequently, introducing antiseptic practices including hand washing before care, sterilization of medical instruments, and wearing of gowns/masks/gloves.[14]

2.2.1 Wound healing mechanisms

There are three categories of wound healing: primary, secondary, and tertiary.[12] Healing by primary intention refers to the closure of a wound in which the edges are closely re-approximated through surgical sutures or tapes.[15] Within 24-48 hrs the epidermal continuity is re-established, by day 5 the incision is filled with granulation tissue and by day 10-14, there is continuous accumulation and proliferation of fibroblasts allowing for minimal scaring[12] and risk

of infection.[15] Healing by secondary intent is characterized by a greater loss of tissue or are surface wounds that create large defects.[12] Secondary intent wound healing requires the wound to heal by leaving it open allowing for granulation tissue at the margins of the wound for contracture and epithelization.[12,15] This category of wound healing is more complex as it requires a greater volume of granular tissue and wound contracture forming a large scar, increased risk of infection as there is no epidermal barrier, and potentiate inflammatory responses.[12,15] Healing by tertiary intent involves delayed primary closure of a wound after 4-6 days, intentionally interrupting secondary intention to mechanically close the wound.[12] Tertiary intent is used after granulation tissue has formed[12] or if the wound site is infected.[15]

2.2.2 Acute vs chronic wounds

Additionally, wounds can be classified as acute or chronic wounds depending on their ability to adhere to the cascade of wound healing phases. Acute wounds are sudden injuries to the integument that follow the normal phases of wound healing, leading to predictable recovery and the restoration of both function and anatomy at the wound site.[12] Examples of acute wounds include minor abrasions, lacerations, surgical incisions, puncture wounds, and skin tears. Conversely, abnormal wound healing occurs when the phases of the wound healing cascade fail to progress in an orderly and timely manner, leading to the formation of chronic wounds (as shown in Figure 2.2.1). [12,16] Examples of chronic wounds include, vascular ulcers, diabetic ulcers, pressure ulcers,[17] advanced stage burn wounds,[18] and septic wounds.[19] Chronic wounds pose several burdens, including the physical, social, and emotional effects on a patient's lifestyle, high treatment costs, prolonged care, and productivity loss in the workforce leading to economic burden.[20] As a result, they present as a significant challenge for both patients and healthcare practitioners.[12,16]

The pathology of chronic wounds include prolonged and exaggerated inflammatory phase observed in figure 2.2.1b, persistent infections, and preventing of dermal and epidermal cellular response to wound healing stimuli.[21] The pathogens associated with wound infection of chronic wounds are *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa (P. aeruginosa*), *Klebsiella pneumoniae, Streptococcus pyogenes* (*S. pyogenes*), *Proteus* spp., *Streptococcus* spp., and *Enterococcus* spp.[22] Infected chronic wounds are often polymicrobial, involving several species of bacteria. For example, *S. aureus* and *P. aeruginosa* have the ability to co-exist in a biofilm becoming more tolerant to antibiotics, therefore a co-infection of these two bacterial species is more virulent than a single infection.[23,24] Chronic wounds fall into several categories namely diabetic ulcers, pressure

ulcers,[17] ulcers secondary to venous hypertension, advanced stage burn wounds,[18] and septic wounds[19] covering a broad-spectrum of wounds.

Especially challenging are diabetic ulcers as diabetic wound healing is prevented by inadequate neoangiogenesis, increased oxidative stress, irregular inflammatory response, peripheral neuropathy, and abnormal apoptosis.[25] The irregular inflammatory response associated with diabetic ulcers is as a result of activation of NF-κB and nod-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome.[26] The high glucose levels associated with diabetes mellitus results in poor circulation responsible for reduced blood flow to the wound site and extremities causing chronic hypoxia, inadequate neoangiogenesis, and increased oxidative stress.[27] Furthermore, diabetic ulcers are susceptible to infection due to this decreased blood flow, neuropathy, and inadequate immune response.[28] Inadequate immune response in diabetic patients is characterised by increased proinflammatory cytokines, T-lymphocyte apoptosis, decreased functioning of polymorphonuclear cells, inhibited fibroblast proliferation, and damaged basal layer of keratinocytes preventing epidermal cell migration.[29] The high blood glucose levels in diabetic patients provides an optimal environment for aerobic Gram-positive *cocci* and haemolytic *streptococci*.[30] As a result diabetic ulcers are highly susceptible to polymicrobial infections comprised of both Gram-positive such as *E. coli*, *Proteus* spp, *Enterobacter* spp, and *Citrobacter* spp, and Gram-negative bacterial strains such as *Pseudomonas* spp and *S. aureus*.[31] The polymicrobial nature of diabetic ulcers leads to the formation of biofilms responsible for antimicrobial resistance. Biofilm infections are responsible for peripheral neuropathy, anatomical damage in extremities/wound site, and trauma expediting the breakdown of the surrounding tissue causing reoccurring infections ultimately prolonging wound healing.[32,33]

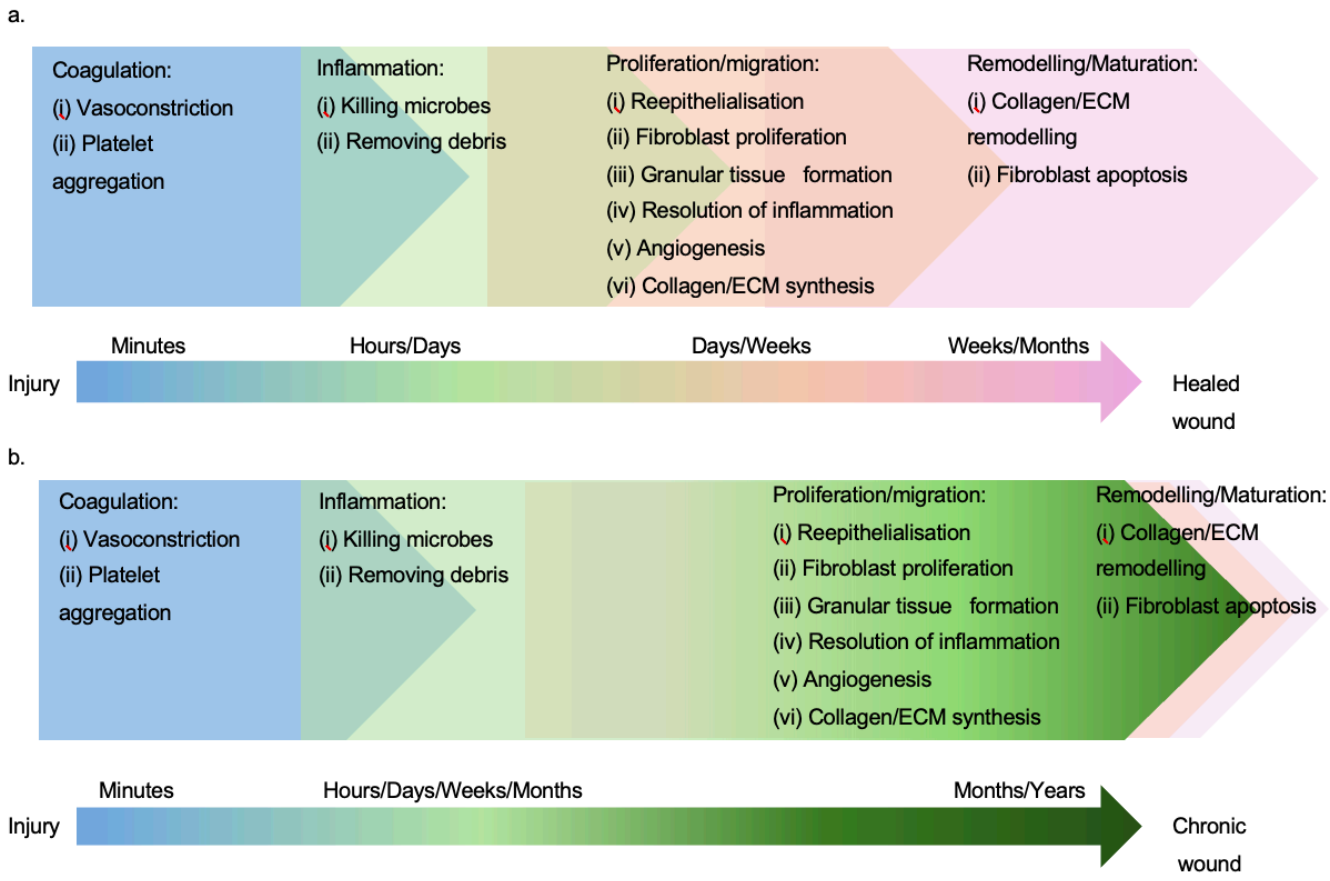


Figure 2.2.1. The wound healing cascade phases and appropriate duration of healing in (a) acute wounds and (b) chronic wounds. The sequence of overlapping, interacting wound healing phases fails to progress in (b) chronic wounds due to failure to inflammation.[44] Adapted from [44]

In 2023, the incidence of acute wounds in the U.S. was reported as 3.5 per 1,000 individuals. The most common types included surgical wounds (110.3 million cases), trauma wounds (1.6 million cases), and abrasions (20.4 million cases).[34,35] Chronic wounds affect an estimated 1% to 2% of the global population, with their treatment accounting for 3% to 5.5% of total healthcare expenditures in developed countries.[36] In the U.S. alone, approximately 8.5 million people were affected by chronic wounds in 2023, resulting in an estimated $28 billion in healthcare costs.[34,35]In 2021, studies in the United Kingdom and Denmark identified 3–4 individuals per 1,000 sustaining one or more wounds, with 15% failing to heal after one year.[16] A study in rural Cote d'Ivoire highlighted the significant burden of skin wounds in sub-Saharan Africa, reporting that 70.7%of wounds were acute while 7.4% were chronic (lasting over four weeks). Notably, 35.5% of chronic wounds had remained untreated for years.[37] Similarly, research at Bingham University Teaching Hospital in northern Nigeria examined 85 patients over an unspecified period, revealing that 70 had acute wounds and 9 had chronic wounds. The study found a significant correlation between age and wound type, indicating that older patients had a higher likelihood of developing chronic wounds.[38]

Systemic factors affecting wound healing include age, stress, and diabetes. The wound healing cascade is dictated by age related characteristics such as enhanced platelet aggregation, increased secretion of inflammatory mediators, delayed infiltration and impaired function of macrophages and lymphocytes, delayed re-epithelialisation and angiogenesis, and reduced collagen deposition, turnover and remodelling. Psychological stress presents with unhealthy behavioural responses such as depression, anxiety, smoking, alcohol consumption, lack of sleep, and poor nutrition.[12] Wound healing is an energy intensive process that requires macronutrients such as carbohydrates, fats, proteins; and micronutrients including amino acids, vitamins, and minerals. Therefore, meeting caloric requirements (±0.9 kcal/g) is vital for synthesis of proteins required for optimal wound healing.[39] However, stress has a pathophysiological effect at the hypothalamic–pituitary adrenal and sympathetic-adrenal medullary axes deregulating immune response by disrupting the regulation of adrenocorticotrophic hormones, cortisol, prolactin, and catecholamines.[40] Stress further impairs cell mediated immunity by reducing the expression of IL-1α and IL-8 crucial for the initial inflammatory response.[12] Altered tissue regeneration in diabetic patients is attributed to the thickening of the basement membrane decreasing leukocyte migration ultimately trapping inflammatory cells increasing the risk of infection. Furthermore, advanced glycosylation end products, as a result of hyperglycaemia, upregulate oxidative stress and inhibit collagen degradation.[41]

Local factors affecting wound healing include oxygenation, foreign bodies, venous insufficiency, and infection.[12] Oxygen delivery is crucial for wound healing as temporary hypoxia produces cytokines that promote cell proliferation, migration, chemotaxis, and angiogenesis whilst chronic hypoxia causes chronic wounds.[42] The foreign body reaction enhances chronic inflammation through the accumulation of exudate at the wound site, infiltration of inflammatory cells, and the formation of granulation.[12] Venous insufficiency is reflected in barrier surrounded capillaries decreasing effective diffusion of oxygen and important nutrients to surrounding tissue.[43] As mentioned previously, cutaneous wound healing is a highly synchronized process, however this process can be disrupted due to infection. Infectious barriers such as devitalised tissue, proteinaceous exudates, and microorganisms impair wound healing through the production of toxins and destructive enzymes, release of free radicals, and degradation of growth factors. Subsequently, down-regulating immune response, decreasing local oxygen levels, localizing thrombosis, releasing vasoconstricting metabolites, disrupting collagen formation and degrading MMP's.[12]

2.2.3 Wound healing phases

Cutaneous wound healing is the process whereby the integument repairs itself following injury (secondary intent).[44] Cutaneous wound healing is a highly synchronized process consisting of overlapping phases of haemostasis, inflammation, proliferation, and remodelling (wound healing cascade).[12] These phases are initiated after injury has occurred and begins with the haemostasis phase. This phase is characterised by vasoconstriction and blood clotting limiting blood loss due to injury. The inflammation phase predominantly consists of neutrophils and macrophages which prevent contamination with invading pathogens and cleanses the wound of non-viable tissue through phagocytosis. The proliferation phase entails tissue granulation, angiogenesis, and epithelialization followed by the last remodelling phase characterized through the transition of the provisional cellular matrix into organized collagen bundles.[20] These wound healing phases, and their associated cellular components are represented as schematic diagrams in Figures 2.2.2-2.2.4.

2.2.3.1 Haemostasis phase

The haemostasis phase (Figure 2.2.2) involves the initial adhesion of platelets at the site of endothelial lesion through interactions between platelet glycoprotein (GP), Ib-IX-V-receptor, and collagen-bound von Willebrand factor (vWF). This loose connection is essential for slowing down platelets in circulation, enabling tight adhesion of platelet receptors α2β1 and GPVI to subendothelial collagen, subsequently, promoting platelet activation via the FcRγ-chain mechanism and the formation of a platelet plug. Platelet activation and aggregation is further amplified through the release of platelet derived granular mediators such as vWF, fibrinogen, P-selectin, adenosine diphosphate, calcium, and serotonin.[17] The rise in systolic calcium increases platelet GPIIb/IIIa receptor affinity[10] for endothelial vitronectin, vWF and soluble fibrinogen binding. The formation of platelet GPIIb/IIIa complexes enables cytoskeleton interactions with cytoskeletal proteins such as talin and kindlin-3, essential for sufficient platelet spreading, stable thrombus formation, and clot retraction.[17,45]

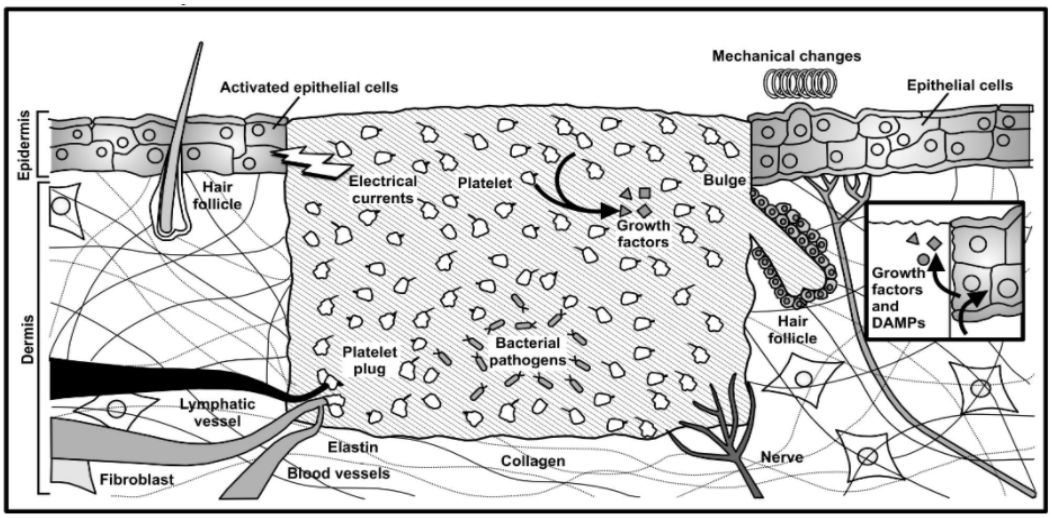


Figure 2.2.2. A schematic diagram of the initial haemostasis phase within wound healing as an immediate response at the wound site depicting the platelet plug and growth factors such as platelet derived growth factor (PDGF). With permission from Prof. MJ Bester (MJB).[45]

Moreover, endothelial lesion simultaneously triggers the coagulation cascade producing thrombin.[46] Thrombin is a clotting enzyme of the serine protease class activated by prothrombin in the coagulation cascade. Thrombin enzymatically cleaves the fibrinopeptides of fibrinogen to form the fibrin monomer.[47] These fibrin monomers spontaneously generate fibrin oligomers which lengthen and form double-stranded protofibrils. Lateral and longitudinal aggregation of the protofibrils yields a three-dimensional (3D) gel network. The 3D fibrin network is further stabilized through covalent crosslinking mediated by Factor XIIa to form a mature fibrin clot.[48]

Furthermore, the α granules of platelets contain PDGF, vascular endothelial derived growth factor (VEGF), endostatin, thrombospondin, and basic fibroblast growth factor (bFGF) which aid in proliferation of smooth muscle cells in the vascular cell walls during angiogenesis.49 Other angiogenic growth factors and cytokines include; tumour necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), and angiopoietins.50 PDGFRα and PDGFRβ are receptor tyrosine kinases that bind to PDGF isoforms (PDGF-BB) stimulating the division of smooth muscle cells and fibroblasts.[51] When PDGF-BB is bound to PDGFRβ it increases pericyte recruitment and induces mesenchymal cell proliferation as a pro-angiogenic effect.[17] VEGF receptors responsible for regulating angiogenesis include, VEGFR-1 and VEGFR-2. Activation of VEGFR-2 occurs through the binding of VEGF isoforms causing homo- and hetero-dimerization of the VEGFR-2 receptor, activating the TSAd-Src-PI3K-PKB/AKT signalling pathway. The resulting AKT from this signalling pathway inhibits the Bcl-2 associated death promoter and caspase 9 apoptotic activity to promote endothelial cell survival. VEGFR-2 binding further results in the activation of ERK in the LCγ-PKC-Raf-MEK-

MAPK signalling pathway, allowing the ERK to bind to transcription factors in the nucleus of endothelial cells whereby it induces gene expression and subsequent proliferation of endothelial cells during angiogenesis.[52] Once bleeding is controlled through hemostasis, the body shifts its focus to removing pathogens and damaged tissue, initiating the inflammatory phase.

2.2.3.2 Inflammatory phase

The haemostasis phase is followed by the inflammatory phase (Figure 2.2.3). The presence of inflammatory cells at the wound site aids in clearing debris and bacteria whilst laying a foundation for keratinocyte proliferation.[53] P-selectin released from activated platelets binds to P-selectin GP ligand-1 on neutrophils, monocytes, and eosinophils causing neutrophil and macrophage recruitment to vascular lesions.[17] Further neutrophil recruitment occurs through damage-associated molecular patterns or pathogen-associated molecular patterns identified by macrophages, dendritic cells, and the endothelium.[54]

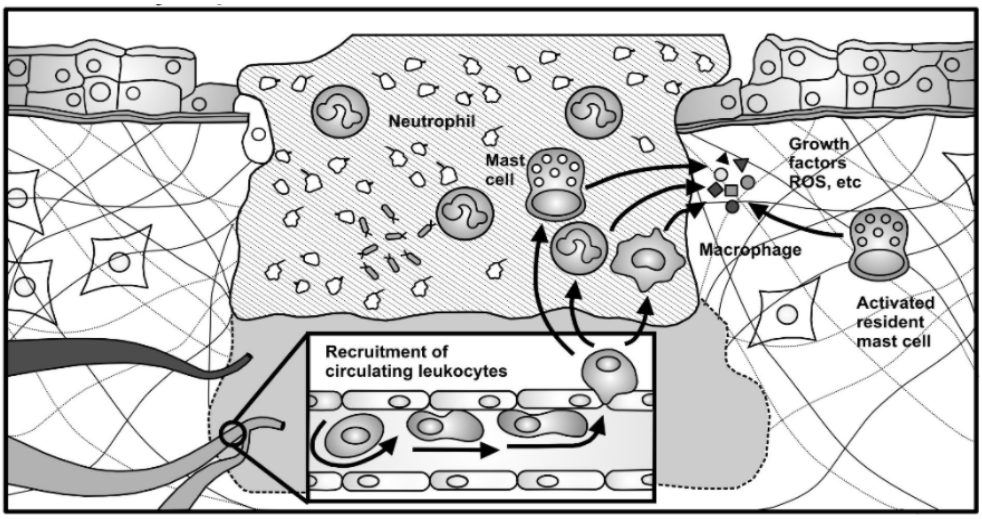


Figure 2.2.3. A schematic diagram of the inflammatory phase of wound healing depicting several cellular role players such as neutrophils, mast cells, and macrophages. With permission from MJB.[45]

Both neutrophils and macrophages are phagocytic cells which contain receptors for lectins, integrins, and opsonin receptors initiating downstream signalling inducing actin polymerization to allow for the extension of pseudopodia used to engulf invading microbes.[55] Neutrophils produce and store cathepsin G, defensins, lysozyme, protease-3, and matrix metalloproteinase-8 (MMP-8) to be released into the phagolysosome, via degranulation, to exert an antimicrobial effect. In addition to phagocytosis, neutrophils can remove invading microbes through release of neutrophil extracellular traps (NETs).[56] NETs formation is stimulated by neutrophil released reactive oxygen species (ROS) through oxidative bursts,[57] and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase conversion of molecular oxygen to a superoxide radical.[58] NETs contain modified chromatin associated with

antimicrobial proteins of the granules, cytoplasm, and nucleus, allowing for extracellular microbial degradation and death. Due to the cellular components of which NETs consist of, subsequent neutrophil cell death is expected post NET formation, also known as NETosis.[58] Once NETs have been released apoptotic neutrophils are ingested by macrophages inhibiting pro-inflammatory signals released by neutrophils, therefore reducing the inflammatory response, and initiating subsequent phases of wound healing.[59] Moreover, macrophages are a part of the innate immune system, however, aid in the transition between innate and adaptive immunity during inflammation. These cells express major histocompatibility complex class II molecules on their membranes, meaning, macrophage engulfed microbes are digested, and associated antigens are expressed on the outer plasmalemma to present these antigens to T lymphocytes. Interaction of T lymphocytes with these surface antigens allows for cytokine release from T lymphocytes activating B lymphocytes for the synthesis of antibodies against macrophage surface antigens.[60] The macrophages are further responsible for the release of growth factors and cytokines recruiting fibroblasts, keratinocytes, and endothelial cells.[20] As inflammation subsides and immune cells clear the wound of debris, the body begins the proliferation phase, where new tissue formation accelerates to restore the damaged area.

2.2.3.3 Proliferation remodelling phases and scar formation

Cellular proliferation and subsequent remodeling phases (Figure 2.2.4) involve fibroblasts which are important cellular components in the proliferation and remodelling phases of cutaneous wound healing; playing key roles in the remodelling of a new extracellular matrix (ECM), production of cellular components within the ECM, and wound contraction[61] with subsequent wound coverage.[62] The ECM is a non-cellular component of all tissues and organs which acts as a cellular scaffold whose functions include tissue homeostasis.[63] Fibroblasts migrate to the wound site and begin proliferation in response to cytokines and growth factors such as PDGF, TGF-β and bFGF secreted by platelets and macrophages.[53] Fibroblasts further respond to TGF-β by differentiating to myofibroblasts[64] which serve two main functions (i) to contract and reduce wound size, and (ii) to secrete ECM proteins.

Due to the presence of contractile cytoplasmic microfilament bundles associated with non-muscle myosin, myofibroblasts generate contractile forces reducing the size of the wound. Myofibroblasts can synthesise collagen types I–VI and XVIII, GP's, and proteoglycans essential for ECM remodelling during wound repair.[65]

Furthermore, keratinocytes are key cellular role players during the re-epithelialization phase of cutaneous wound healing. Keratinocytes located at the wound edge migrate and proliferate

during wound repair. Keratinocyte proliferation is stimulated by epidermal growth factor (EGF), TGF-α, heparin binding EGF, and bFGF secreted from platelets, macrophages, and dermal fibroblasts. Keratinocyte migration is stimulated by loss of tension in cell attachment points in the basal lamina. These cells are at the forefront of migration and are responsible for dissolving the fibrin clot through the binding of urokinase-type plasminogen activator (uPA) to its receptor, uPAR.[53] This receptor is upregulated on migrating keratinocytes to maximize release of plasmin, a fibrinolytic enzyme, as a result of uPA-uPAR binding.[66] Plasmin therefore degrades the fibrin clot, subsequently facilitates keratinocyte migration, regulates growth factor synthesis, and activates MMPs assisting in damaged ECM degradation and remodeling.[67]

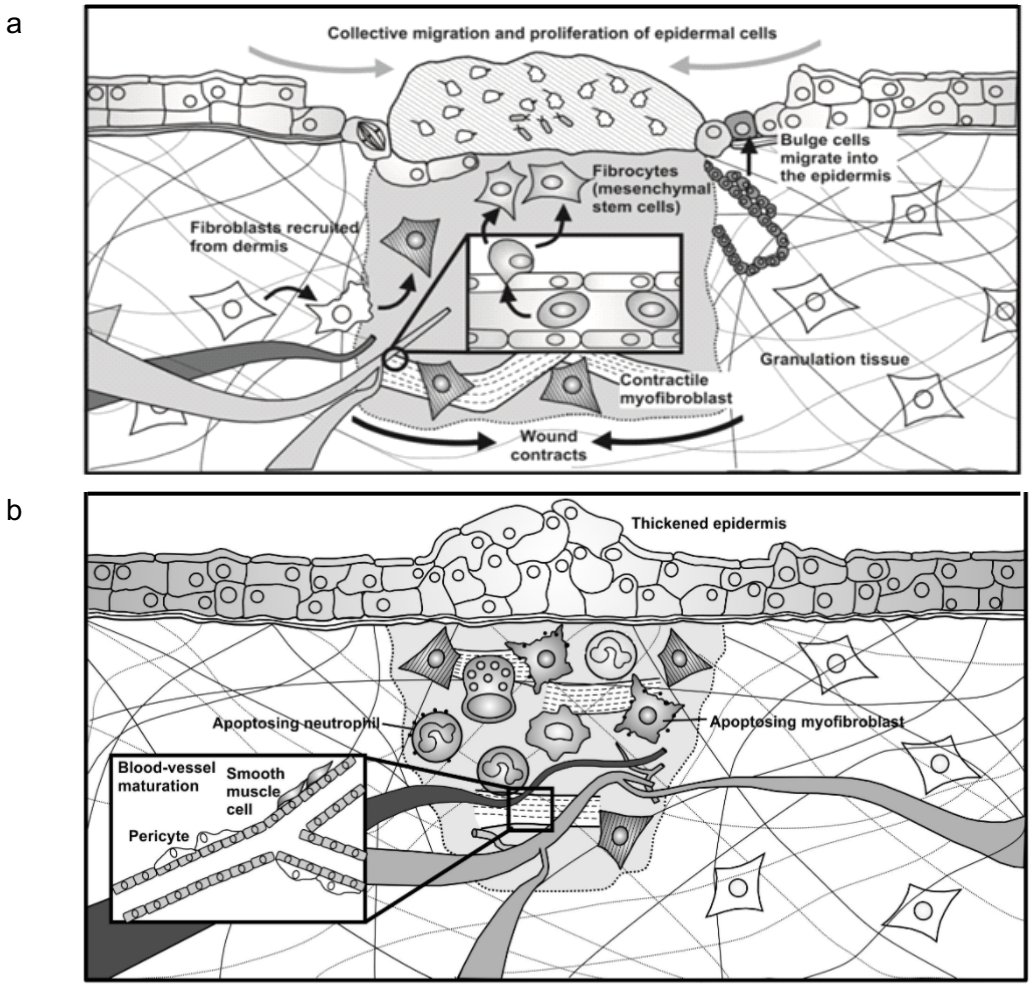


Figure 2.2.4. A schematic diagram of (a) cell migration and the subsequent tissue remodelling involving collagen deposition with an observed reduction of inflammatory processes and completed angiogenesis. With b) scar formation, the epidermis is thickened and the neutrophils and myofibroblasts undergo apoptosis. With permission from MJB.[45]

During the proliferation phase, the development of new blood vessels—angiogenesis—becomes crucial, ensuring adequate oxygen and nutrient supply to support tissue regeneration and wound closure. As mentioned previously failure to adequately complete these phases of wound healing in a timely manner often results in chronic wounds that require extensive treatment.

2.2.4 Treatment of chronic wounds

Chronic wounds heal through mechanisms distinct from those of acute wounds, leading to prolonged healing times and extended treatment plans.[68] These wounds often become stuck in a particular phase (typically the inflammatory phase) or repeatedly cycle between the inflammatory, proliferative, and remodelling phases without achieving complete healing.[69] Chronic wound beds frequently contain a damaged extracellular matrix, inflammatory enzymes, and senescent cells, necessitating wound debridement. However, caution must be taken to avoid removing newly formed or healthy tissue.[70] Surgical debridement, being non-selective, may also remove viable tissue, prompting some clinicians to prefer autolytic debridement, the use of proteases and collagenases in the wound fluid, as well as natural moisture to rehydrate, soften, and liquefy non-viable tissue.[71]

In chronic wounds, wound exudate impairs cellular proliferation and angiogenesis by containing high levels of matrix metalloproteinases, which degrade matrix proteins, growth factors, and cytokines.[72,73] Therefore, an optimal dressing should maintain a moist environment, absorb excess exudate, prevent maceration of surrounding tissue, and block bacterial infiltration.[70] For cases involving poor blood circulation, compression stockings or bandages should be used, while offloading strategies are essential for managing diabetic foot and pressure ulcers to relieve abnormal pressure points.[74]

Table 2.2.1. Treatment options for chronic and infected wounds healing by secondary intent. Adapted from Parkar *et. al*.[69]

Category	Product	Key benefits	Drawbacks	Indications
Secondary intent wounds	Hydrocolloids	Autolytic debridement, waterproof, self-adherent, reduces pain	Odor formation, may adhere to wound bed, can cause overgranulation	Clean wounds, ulcers (not for heavily exuding wounds)
	Alginates	Highly absorbent, antimicrobial,	Requires frequent changes, may dry	Heavily exuding,

		promotes hemostasis, non-adherent	out wound bed, distinctive odor during dressing changes	bleeding wounds (not for dry wounds)
	Hydro-conductive dressings	Removes slough and bacteria, retains fluid, promotes wound healing	Requires secondary dressing, not for arterial bleeding, frequent changes needed	High-exudate wounds, chronic wounds
	Gauze	Readily available, effective for packing, can be impregnated with antimicrobials	Can stick to wounds, requires frequent changes, may disrupt wound healing	Necrotic, infected wounds, burns, ulcers
	Foam	Absorbent, waterproof, non-occlusive, reduces friction risk	May require secondary dressing, can macerate surrounding skin, some foams are not conformable	Under compression, burn wounds, moderately exuding wounds
	Hydrogels	Highly absorbent, antimicrobial, conforms to wound, maintains moisture	Requires secondary dressing, not suitable for dry wounds, can stick to wound bed	Ulcers, burns, necrotic wounds, infected wounds
	Transparent films	Impermeable to bacteria, protects from friction, maintains moisture	Painful removal, may cause maceration, not absorbent	Partial-thickness wounds, cover for other dressings, abrasions
Advanced treatment	Protease modulating matrix	Removes proteases, maintains wound healing environment,	Not for infected wounds, requires hydration before application, frequent	Diabetic foot ulcers, pressure ulcers, wounds with minimal exudate

		promotes granulation	assessment needed	
	Enzymatic dressings	Selective enzymatic debridement, fast-acting, painless	Expensive, frequent use needed, can cause maceration	Sloughy wounds, necrotic wounds
Infected secondary intent wounds	Iodine dressings	Broad-spectrum antimicrobial, removes biofilm, promotes autolysis, conformable	Microbial resistance risk, iodine allergy risk, long-term use can delay healing	Critically colonized wounds, moderate-to-heavily exuding wounds
	Silver dressings	Inhibits bacteria (including resistant strains), cost-effective, maintains moist healing environment	Requires secondary dressing, may stain skin, contraindicated in dry wounds	Draining wounds, ulcers, donor sites, dehisced wounds
	Chlorhexidine	Broad-spectrum antibacterial, non-adherent, effective against Gram-positive and Gram-negative bacteria	Ineffective against Pseudomonas, not for critically infected wounds, limited absorption	Superficial wounds, surgical sites, infection prevention
	Hydrophobic dressings	Traps bacteria and fungi, prevents endotoxin release, available in various forms	May require secondary dressing, odor retention from trapped bacteria, not for dry wounds	Cavity wounds, exuding wounds, moderate-to-heavily exuding infected wounds

Therefore, chronic wounds are difficult to treat and alternative therapies are being developed, including the use of medicinal honey based products as seen in table 2.2.1.

2.3 Medicinal honey

Public interest in the therapeutic use of natural honey has greatly increased, due to this demand, licensed medical grade honeys are available. Synergistic treatments with medicinal honey and chronic wound products for wound treatment are typically used as a honey-coated or impregnated topical dressing.[6] Table 2.3.2 outlines the commercialised honey based wound dressing products and their intended use adapted from Yasin *et. al.*[75]

Table 2.3.1. The intended use and therapeutic effect of several commercialised honey-based products for wound application, adapted from Yasin et. al.[75]

Dressing Type	Product Name	Origin	Type of Wound	Therapeutic Effect
Hydrocolloid Dressing	MediHoney®[76]	*Leptospernum scorparium*	Chronic, acute, burn wounds	- Reduce healing time - Increase tissue growth
Film Dressing	TheraHoney®[77]	*Leptospernum scorparium*	Several types of ulcers, acute, traumatic and burn wounds	- Provides moist environment - Reduce pain and inflammation - Antimicrobial activity
Foam Dressing	Actilite®[78]	*Leptospernum* dervived	Several types of ulcers, acute, traumatic and burn wounds	-Antimicrobial activity - Provides a moist environment
Alginate Dressing	Algivon®[79]	*Leptospernum* derived	Several types of ulcers and acute wounds	- Antimicrobial properties - Absorbs excess exudate
Mesh Dressing	Activon®[80]	*Leptospernum* derived	Several types of ulcers, acute, traumatic and burn wounds	- Provides a moist environment - Absorbs excess exudate - Reduce inflammation - Antimicrobial activity

A study conducted by Robson *et. al*[81] used MediHoney® to treat 105 patients suffering with various wound types, patients received either a conventional wound dressing or antibacterial MediHoney® treatment. [81] The healing rate of the MediHoney® group at 12 weeks was equal to 46.2% compared to the 34.0% in the conventional group, with a 12.2% increase in healing rates of the MediHoney® group. [81] Over one year, 70 patients underwent free tissue reconstruction, with 56 (80%) consenting to randomization and 49 (70%) successfully randomized (25 to MediHoney® wound dressings, 24 to conventional dressings).[82] Wound swabs were positive in 36% of the honey group and 38% of controls, with MRSA detected in 28% and 25%, respectively; 38% of these cases required intervention.[82] Hospital stays were significantly shorter in the honey group (median 12 days, IQR 10–21) than in controls (median 18 days, IQR 13–28; $p<0.05$), while dressing costs remained comparable.[82] The study confirmed the feasibility of an randomised clinical trial, suggesting that a larger sample size is needed to establish clinical benefits. This study confirmed that further research is needed to confirm a shorter duration of hospital admission and establish whether this is due to more

rapid healing.[82] Additionally, A study conducted by Lund-Nielson *et. al.*[6] treated 69 cancer patients with malignant wounds, group A was treated with Algivon® and Activon® honey-coated bandages while group B with silver-coated bandages.[6] No statistically significant difference was noted between the groups with respect to wound size, degree of cleanliness, exudation, malodor, and wound pain. Indicating a similarity of activity between the two interventions.[6] However, there was a median decrease in wound size of 15 cm² and 8 cm² in group A and B, respectively ($p = 0.63$), indicating an increased wound closure rate of malignant wounds in cancer patients when treated with Algivon® and Activon® honey-coated bandages.[6]

One of the most prominent limitations for all the abovementioned studies is the small sample sized used when conducting research on honey-coated dressings. A small sample size in clinical trials can increase result variability, reduce statistical power, and make it harder to detect true effects. It additionally, raises the risk of false positives or negatives, and decreases confidence in the outcomes due to random data fluctuations, complicating the accurate evaluation of a treatment's efficacy.[83] There is a need for conducting large scale randomised clinical trials to standardise wound healing measurements (e.g., wound closure rates, infection rates, and pain reduction). This would assist in understanding the long-term effects and adverse events on diverse patient populations.[84]

Whilst clinical trials involving the wound healing activity of honey based wound dressings indicating their ability to promote wound healing, these trials do not investigate the exact molecular pathways responsible for the antimicrobial, anti-inflammatory, and tissue regenerative properties of these treatments.[84] Subsequently, most studies focus on general observations like reduction in bacterial load or wound closure time, rather than elucidating specific cellular and molecular mechanisms.[85] The scope of research could be broadened to investigate the interactions between medicinal honey products with immune cells and epithelial cells.[85] With focus on identification of the bioactive compounds responsible for each therapeutic effect.[85] Additional exploration could include researching the influence medicinal honey products have on cytokines and growth factor expression in wound healing.[85]

The synergistic activity of honey allows for sterilisation of the wound, stimulation of tissue growth, epithelialisation, and reduced scar formation all of which contribute to the four stages of wound healing.[75] During the inflammatory phase, honey stimulates monocytes to release inflammatory cytokines that initiate and amplify the inflammatory process. Simultaneously, stimulating neutrophils, macrophages, and phagocytosis to remove debris and bacteria from the wound site.[86] Honey is known to have a pH between 3.24 to 6.1,[75] therefore increasing the

acidity of the wound, initiating oxygen release from haemoglobin creating an unfavourable wound environment for destructive proteases and anaerobic microoganisms.[1,87] The decrease in several protease activities enhances macrophage and fibroblast infiltration; known to eradicate bacteria and inhibit biofilms during the inflammatory phase. Whilst promoting growth of new granular tissue and epithelialisation during the proliferation phase.[86,88]

The healing properties of honey are further related to the high viscosity and thick consistency which makes it a suitable topical treatment, acting as a physical barrier between the wound site and the external environment protecting the wound and preventing further pathogenic infiltration at the wound site.[1,75] Modern medicine has drawn attention to the use of natural products with strong antimicrobial properties such as honey and other combination therapies.[75] Table 2.3.2 represents the benefits of honey based treatment for wound healing in comparison to other bioactive compounds adapted from Yasin *et. al*.[75]

Table 2.3.2. A comparison between honey and other bioactive compounds used in the treatment of wounds adapted from Yasin *et. al.*[75]

Bioactive Compounds	Benefits	Shortcomings
Honey	-Rapid epithelisation and contraction - Reduce pain and inflammation - Cost-effective	- Stinging sensation and discomfort - Low stability - Short half life
EGF	- Maintains tissue homeostasis	- Restrict absorption at the wound site - Exudate removal before reaching wound site - Uncontrolled cell invasion and growth - High cost
ECM Protein	- Regulate cell differentiation, migration, and proliferation	- Impaired apoptosis - Dysfunction of skin abilities
Silver sulfadiazine	- Prophylactic treatment for wound infection, particularly for second- and third-degree burns	- Toxic to fibroblasts at large concentrations

Honey based dressings are classified as medical devices, regulated by the US Food and Drug Administration, European Medicines Agency, National Medical Products Administration, Therapeutic Goods Administration, Health Sciences Authority, and Medical Device Authority.[75] Medical grade honey is commonly derived from the *Leptospermum spp* found in New Zealand and Australia,[90] *Cliftonia monophylla* in the USA, *Kunzea ericoides* in New Zealand, Maleleuca cajupati in Malaysia, *Salvis officinalsis*, and *Aronia melanocarpa* in England.[11]

2.4 Manuka honey

A type of medicinal honey from New Zealand rich in *Leptospermum scoparium* shrub nectar, Manuka honey, has been registered for use in medicinal products known as Manukaguard and Medihoney[1] due to its unique antibacterial activity. In contrast to many other honey types, the antimicrobial activity is termed "non-peroxide" as H_2O_2 plays a minimal role as an antimicrobial factor.[91] MGO is the dominant component associated with non-peroxide based antimicrobial properties,[87,88] however, glyoxal (GO), leptosin, and various phenolics synergistically modulate non-peroxide activity.[92] Various studies have quantified MGO content in Manuka honey using the agar diffusion "phenol equivalence" assay, showing a strong correlation between MGO content and antibacterial activity.[91] Results obtained include total activity, H_2O_2 and non-peroxide activity used to quantify antibacterial activity remaining when H_2O_2 is removed,[91] this is referred to as the Unique Manuka factor (UMF).[90] The market value and quality of medicinal grade Manuka honey is correlated with the UMF.[92]

MGO is derived from the non-enzymatic dehydration of dihydroxyacetone in aqueous solutions.[93] Dihydroxyacetone occurs at high concentrations in the Manuka nectar source,[94] whilst MGO is not present, indicating that MGO concentrations increase as honey matures during transport and storage.[93] High concentrations of MGO elicit strong non-peroxide based antimicrobial effects that can withstand substantial dilution with wound exudate.[87,88] The observed antimicrobial effects of Manuka honey include the inhibition of methicillin resistant *S. aureus* (MRSA), *S. pyogenes*, *E. coli*, and *P. aeruginosa* preventing subsequent biofilm establishment at the wound site.[88]

The antimicrobial mechanism of action against the various bacterial strains may be through disruption of cell division, impaired structural integrity, and reduction of cellular mobility.[92] A study conducted by Roberts *et. al.*[95] measured a significant reduction flagellum-associated gene expression in *P. aeruginosa* post treatment with 24% (w/v) Manuka honey, impacting both regulatory (fliA, fleN, fleQ and fleR) and structural (fliC and flhF) genes decreasing *P. aeruginosa* invasive virulence.[95] Figure 2.4.1 depicts the different hypothesised mechanisms of action against MRSA and *P. aeruginosa*. During normal cell mitosis MRSA cells duplicate and segregate their chromosomes forming a proteinaceous ring (septa) across the midline of the cell creating two adhered daughter cells.[96] Manuka honey disrupts MRSA cell mitosis by inhibiting the production of peptidoglycan hydrolases preventing degradation of the septa and accumulation of separated cells eventually leading to cell death.[96]

In contrast *P. aeruginosa* can tolerate higher concentrations of Manuka honey, with inhibitory concentrations causing loss of structural integrity.[96] Cell integrity is modulated by the up regulation of a key anchor protein known as outer membrane protein F (OprF). This protein covalently bonds to the outer membrane and peptidoglycan layer maintaining cell homeostasis and shape.[96,97] Following treatment with Manuka there is a decrease in OprF expression and concomitant increase in cell lysis and death.[96]

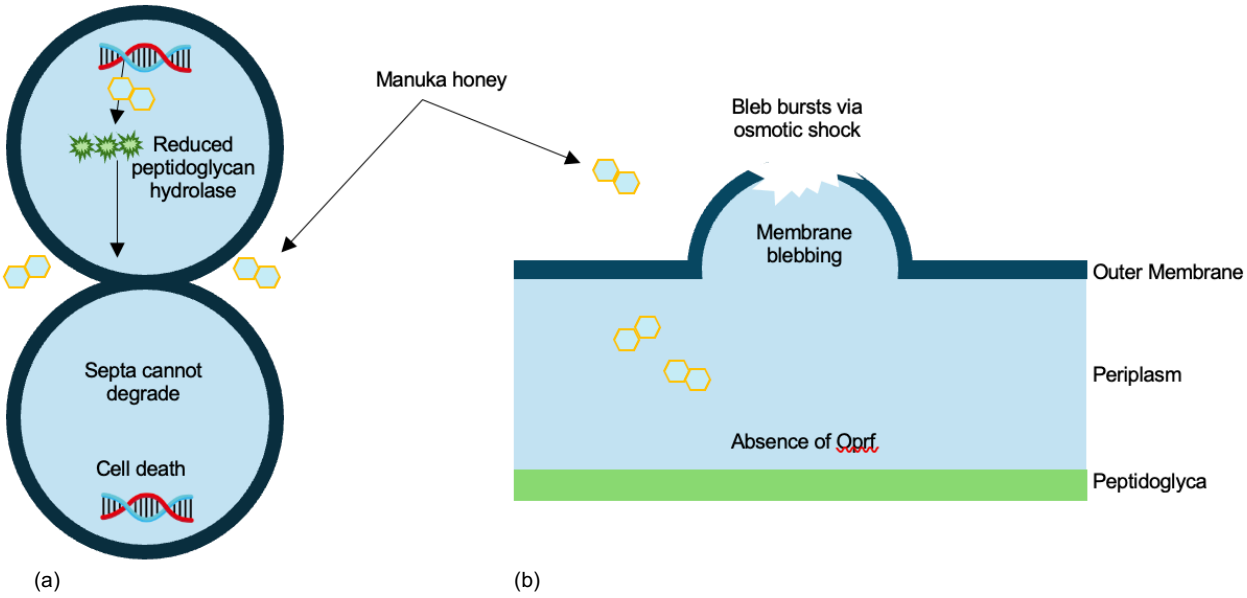


Figure 2.4.1. The hypothesised mechanism by which Manuka honey inhibits (a) S. aureus (MRSA) and (b) *P. aeruginosa*. Where (a) represents late-stage cell mitosis following the formation of the septa and reduction in peptidoglycan hydrolase causing the daughter cells to remain attached eventually leading to cell death, and (b) represents the destabilisation of the cell shape through down regulation of OprF leading to membrane blebbing and cell lysis. Adapted from Jenkins *et. al.*[99]

Further investigation into synergistic combinations of Manuka honey and antibiotics has shown promising combinations for treating wound infections of multi-drug-resistant bacteria.[98] A study done by Jenkins *et. al.*[99] observed improved susceptibility of MRSA to oxacillin when combined with Manuka honey due to disrupted regulation of the mecR1 gene accounting for restored susceptibility.[99] In another study conducted by Müller *et. al.,*[100] *S. aureus* isolates (including MRSA strains) had sustained susceptibility to rifampicin when combined with Medihoney (Medihoney Ltd, Slough, United Kingdom).[100] It is important to note that oxacillin and rifampicin are of different drug classes that inhibit different targets such as the 30 S ribosome, RNA polymerase, and penicillin binding proteins; giving credence to the complex nature of honey with several bioactive components that effect multiple cellular targets.[99]

Manuka honey is known to have a relatively low pH (3.5-4.5), inhibiting microbial growth, stimulating macrophages, and in chronic wounds, reduces protease activity and increase fibroblast activity and oxygenation.[98] A study conducted by Minden-Birkenmaier and Bowlin, using Manuka honey-containing poly (Ɛ-caprolactone) nanofiber scaffolds applied as engineered wound dressings, observed bacteriostatic inhibition of *E. coli* strains and positively induced fibroblast infiltration of the nanofiber scaffolds.[1] Moreover, MGO has immunomodulating properties enhancing wound healing and tissue regeneration[101] these include T and B lymphocyte proliferation; stimulation of phagocytosis, regulation of the synthesis of pro-inflammatory cytokines such as TNF, interleukin-1-β (IL-1-β), IL-6, ROS; and upregulation of prostaglandin E2 and cyclooxygenase-2 (COX-2).[102]

2.5 Buckwheat honey

Buckwheat honey originates from the flowers of the *Fagopyrum esculentum* common Buckwheat plant mainly produced within the United States and Chinese regions.[90,103] This honey type contains minerals such as iron, zinc, and manganese which are more abundant than that found in Manuka honey.[103] A study conducted by Pasini *et. al.*104 suggests the main phenolics in Buckwheat honey include caffeic acid, protocatechuic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, syringic acid, *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and *p*-hydroxybenzoic acid. However, Jiang *et. al.*[105] used high performance liquid chromatography to identify the most abundant phenolics in Buckwheat honey these being benzoic acid, *cis,trans*-abscisic acid, gallic acid, isoferulic acid, methyl syringate, *p*-coumaric acid, ferulic acid, protocatechuic acid, *trans,trans*-abscisic acid, vanillin, 4-yydroxybenzaldehyde, and 4-hydroxy benzoic acid.[105] Buckwheat honey is a dark amber colour with a pungent odour that has a higher polyphenol content than that of Manuka honey identified in table 2.5.1.[103]

A study by Dheng *et. al.*[103] concluded that the higher content of phenolic compounds found in Buckwheat honey is statistically significant ($P<0.05$) to that of Manuka honey. Furthermore, they identified a greater antioxidant capacity of Buckwheat honey and similar antibacterial activity of Buckwheat and Manuka honey.[103] Evidence based research has shown that honeys which are dark in colour have high polyphenol contents which exhibit strong antibacterial and antioxidant activity.[103] This significant increase in polyphenol content found in Buckwheat honey can account for the greater antioxidant capacity.

Table 2.5.1. Physiochemical parameters of Buckwheat and Manuka honeys as identified by Deng *et. al.*[103]

Physiochemical parameter	Buckwheat honey	Manuka honey
Fructose (g/100 g)	36.4 ± 0.14*	33.0 ± 0.17
Glucose (g/100 g)	34.8 ± 0.35*	27.8 ± 0.05
Sucrose (g/100 g)	0.60 ± 0.03*	0.98 ± 0.04
Maltose (g/100 g)	nd	0.42 ± 0.01
Protein (mg/g)	1.83 ± 0.01*	0.63 ± 0.01
Total polyphenol content (mg/kg)	1498 ± 37.3*	561 ± 2.82
MGO (mg/kg)	4.61 ± 0.16*	351 ± 27.3

nd, not detected.

* $P < 0.05$, statistically significant in comparison with Manuka honey

As mentioned previously MGO is responsible for the non-peroxide antibacterial activity of Manuka honey,[87,88] according to Table 2.5.1 the MGO content in Buckwheat honey is significantly lower than that of Manuka honey[103] suggesting a peroxide-based mechanism of action is used to maintain the high antibacterial activity associated with Buckwheat honey. Research done by Brudzynski *et. al.*[106] shows a cause-and-effect relationship between H_2O_2 generated OH radicals and growth inhibition of bacterial cells.[106] Giving credence to earlier research that suggests Buckwheat honey has a substantial correlation between H_2O_2 content and antibacterial activity whilst Manuka honey's antibacterial activity is H_2O_2 independent and correlated to the internal MGO concentration.[106]

An earlier study conducted by Brudzynski *et. al.*[107] found that bacterial cultures exposed to Buckwheat honeys of American origin showed signs of oxidative stress correlated to the generation of OH radicals.[107] The metal-catalysed Fenton reactions requires H_2O_2 as a substrate for OH formation.[106] OH radicals cause DNA/RNA degradation associated with impaired cell proliferation, and protein/lipid peroxidation associated with oxidative injury to cell membranes (effecting permeability) both of which lead to a decrease in bacterial cell viability and subsequent cell death.[106] This research provides evidence that H_2O_2 generated OH radicals are key role players in the bacteriostatic activity of Buckwheat honey,[106] allowing for more research into honeys of other origin.

2.6 Honey constituents

Honey is a highly concentrated and viscous fluid[1] consisting of proteins, amino acids, enzymes, flavonoid and phenolic compounds, vitamins and minerals, water and sugar.[1,102] Sugar accounts for 95-99% of honey dry weight consisting of reducing sugars such as fructose, glucose, maltose, and sucrose.[108] Alongside other disaccharides including isomaltose, maltotriose, melezitose turanose, meli-biose, nigerose, and panose.[108] Fructose

(32.56- 358.2%) and glucose (28.54-31.3%) are derived from the conversion of floral nectar disaccharides and are the most abundant carbohydrates present in honey representing 85-95% of total sugar content.[108,109]

Honey dressings directly affect the osmolarity at the wound site by drawing excess exudate and oedema related fluid out of the wound[87] whilst maintaining wound hydration.[75] The high sugar content in honey generates a strong osmotic gradient that draws fluid through the subdermal tissue. This fluid flow has low water activity flushing bacteria, debris, and necrotic tissue out of the wound during the inflammatory phase, simultaneously, transporting nutrients and oxygen from the subdermal tissue into the wound site. The high osmotic pressure is responsible for holding the wound edges closely together during the proliferation and remodelling phases allowing for minimal scarring.[86] Additionally, the high sugar content found in honey acts as another source of glucose for proliferating fibroblast and endothelial cells within the wound.1 Honey-based dressings need to be changed less often than other types of dressings thereby reducing patient pain and discomfort.[89]

Notably, 4-5% of the total sugar content is attributed to fructooligosaccharides serving as a prebiotic agent.[108] Prebiotics are nondigestible food ingredients that aid in stimulating the growth and activity of several bacterial strains in the colon.[110] Fermentation of fructooligosaccharides in the colon elicit beneficial physiological effects such as increasing faecal weight and calcium absorption, decreasing gastrointestinal transit time and blood lipid levels, and most significantly increasing the population size of bifidobacteria in the colon.[110] The increase in Bifidobacteria is hypothesised to benefit host health by reducing blood ammonia levels, producing vitamins and digestive enzymes, and synthesizing compounds that inhibit pathogenic agents.[110]

The sugars within honey determine the viscosity, crystallisation, thermal, pH, and rheological properties of honey.[109] The wound healing functionality of the sugar components is coupled with the production of gluconic acid; a by-product of water and glucose oxidation by glucose oxidase (GOx) to form H_2O_2 and gluconic acid as seen in figure 2.6.1.[111,112] Gluconic acid is the main organic acid found within honey along with trace amounts of formic, citric and acetic acid.[111] Organic acids constitute 0.57% of total honey weight,108 responsible for the characteristic taste of honey and acidity, maintaining a pH of 3.2-4.5.[111,112] Bacteria grow best in neutral pH levels ranging from 6.5-7.5, therefore the maintained acidity of honey gives credence to the antibacterial activity of the sugars found within honey.[113] Furthermore, when used as a wound dressing honey maintains its low pH, enhancing offloading of oxygen from

haemoglobin in the capillaries, stimulating PDGF leading to fibroblast proliferation, and inhibiting protease activity.[114,115] Increased protease at the wound sight can delay or halt wound healing by destruction of protein fibres and fibronectin necessary for activation and migration of fibroblasts and epithelial cells respectively.[114]

The osmotic potential of honey is increased through the breakdown of sucrose into fructose and glucose via bee-derived invertase as observed in 2.6.1.[112] This osmotic action benefits wound healing by drawing lymph fluid to the surface of the wound from the subcutaneous tissue subsequently removing debris, necrotic tissue, and slough from the wound site.[116]

Moreover, H_2O_2 formed in the GOx cascade is a main component within honey further contributing to the antibacterial activity, it acts as a potent antiseptic sterilising the wound sight and strong oxidising agent against invading microorganisms.[113] The antiseptic effects of H_2O_2 are termed “peroxide base” antibacterial activity stimulating macrophages and VEGF allowing for fibroblast proliferation and angiogenesis.[112,116] H_2O_2 further reacts with bacterial oxygen and iron forming a toxic hydroxyl radical.[116] Therefore, the production of H_2O_2 can be toxic to cellular tissue when saturated,[112] however, catalase (enzyme found in honey) hydrolyses H_2O_2 to water and oxygen maintaining the ROS concentration below the level that causes inflammatory effects.[114,117]

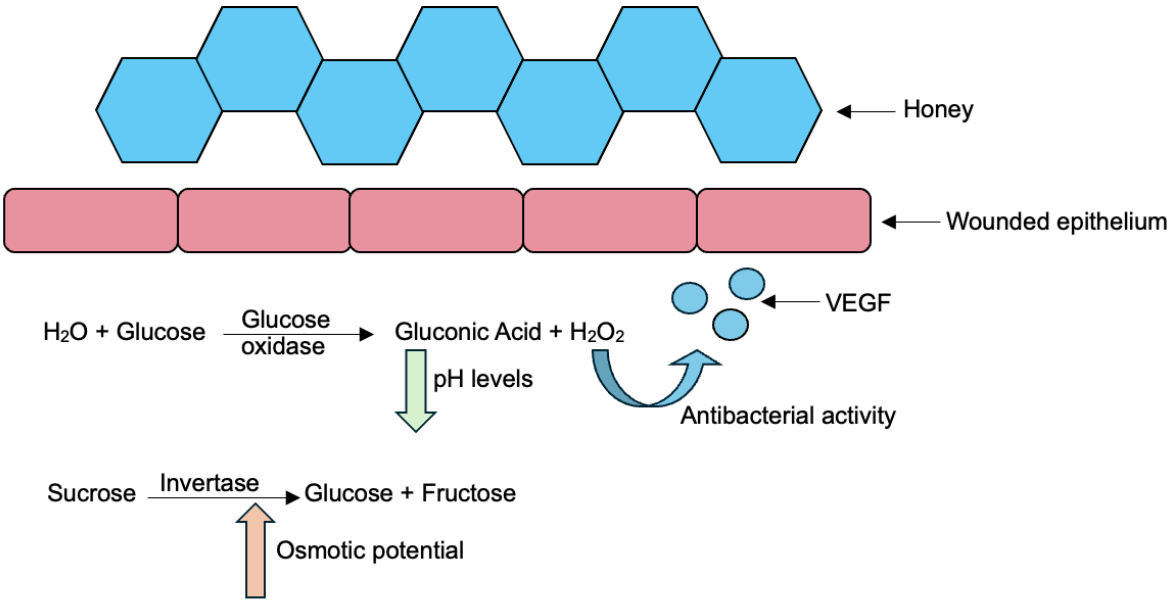


Figure 2.6.1. The fundamental activity of sugars, H_2O_2, and various enzymes found within honey. Adapted from Yasin *et.al.*[75]

Several minerals are found within most honey's, including all major minerals such as phosphorous, sodium, calcium, potassium, sulphur, magnesium, and chlorine.[118] Trace minerals include iron, zinc, silicon, zirconium, and lithium.[108,118] Moreover, honey contains bioactive vitamins such as riboflavin, pyridoxine, tocopherol, vitamin K, niacin, retinol, folate, pantothenic acid, and ascorbic acid.[108,118] Ascorbic acid is the most frequent vitamin found in honey[111], however, the concentration may vary depending on processing and storage of honey, and its apicultural origin.[119] Ascorbic acid is a strong reducing agent capable of rapidly scavenging for ROS (mainly peroxides) acting as an antioxidant, conversely, it is known to have a pro-oxidant antibacterial effect through (i) entering bacterial cells subsequently forming H_2O_2 and other ROS and/or (ii) the generation of lactate and acetic acid.[114,119] Interestingly, the interaction of ascorbic acid with free metal ions could contribute to oxidative damage of bacterial cells through the catalytic production of hydroxyl and alkoxyl radicals.[119]

Moreover, medicinal honey has been widely recognized as a therapeutic treatment due to its anticancer, antidiabetic properties, anti-inflammatory, antioxidant, and antimicrobial bioactivity.[75] The anticancer activity of honey is due to its ability to induce apoptosis and cell cycle arrest in various cancer cell lines, including breast, liver, and colorectal cancers.[120,121] Additionally, honey's modulation of oxidative stress resulting in reduced oxidative stress, thereby preventing DNA damage and inhibiting tumour progression.[120,121] Studies also suggest that honey suppresses cancer metastasis by hindering cell migration and invasion. These effects are attributed to honey's ability to modulate key signalling pathways involved in cancer growth and inflammation.[120,121] However, while in vitro and animal studies provide strong evidence, large-scale clinical trials are needed to confirm its efficacy in human cancer treatment).[120,121] Additionally, the anti-inflammatory and antioxidant capacity of honey is largely due to its phenolic compounds, including flavonoids like quercetin, luteolin, and kaempferol, as well as phenolic acids such as caffeic and ferulic acids.[120,121]

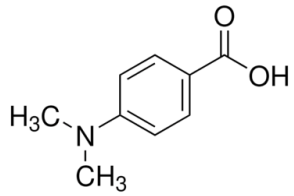
2.7 Honey polyphenols and associated anti-inflammatory and antioxidant activity

The phenolic compounds within honey constitute 56-500 mg/kg of honey with a concentration range of 60-460 mg/100 g of honey.[122] Common phenolic acids present in honey include 4-(dimethylamino) benzoic, caffeic, chlorogenic, *p*-coumaric, gallic, syringic, and vanillic acids (Figure 2.7.1) and fall within two categories, namely, the hydroxycinnamic acids or the hydroxybenzoic acids.[109,118] Hydroxycinnamic acids contain multiple hydroxyl groups used as electron donors to target ROS's.[123] Whilst, hydroxybenzoic acids exert their antioxidant effect due to the position of the OH group in the aromatic ring.[124] Therefore, the antioxidant

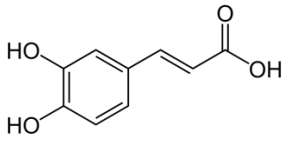
mechanisms of the phenolic compounds include free radical sequestration, metal ion chelation, flavonoid substrate action for hydroxyl/superoxide radical actions, and proton donation.[123] Flavonoids are a family of polyphenols subdivided into different classes.[125] The flavonoid component of honey consists of apigenin, genistein, pinocembrin, pinobanksin, chrysin, galangin, kaempferol, luteolin, and quercetin (as seen in Figure 2.7.1).[109] Flavonoids exert an antioxidant effect due to the highly reactive OH groups allowing for rapid scavenging of ROS's creating a stable, less reactive molecule.[126]

(a) Phenolic acids

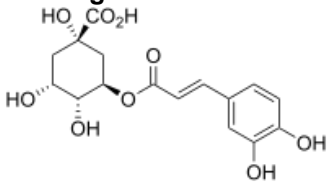
4-(dimethylamino) benzoic acid



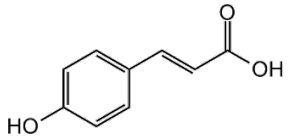
Caffeic acid



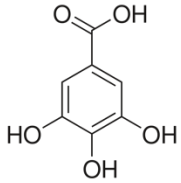
Chlorogenic acid



***p*-courmaric acid**



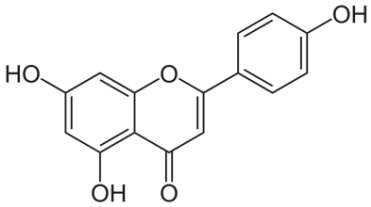
Gallic acid



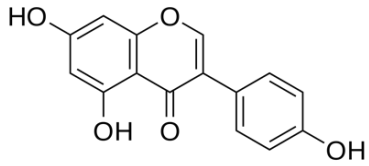
Syringic acid

(b) Flavonoids

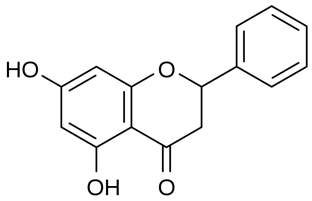
Apigenin



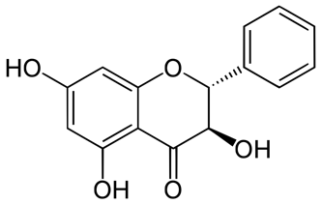
Genistein



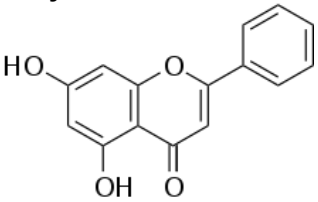
Pinocembrin



Pinobanksin



Chrysin



Galangin

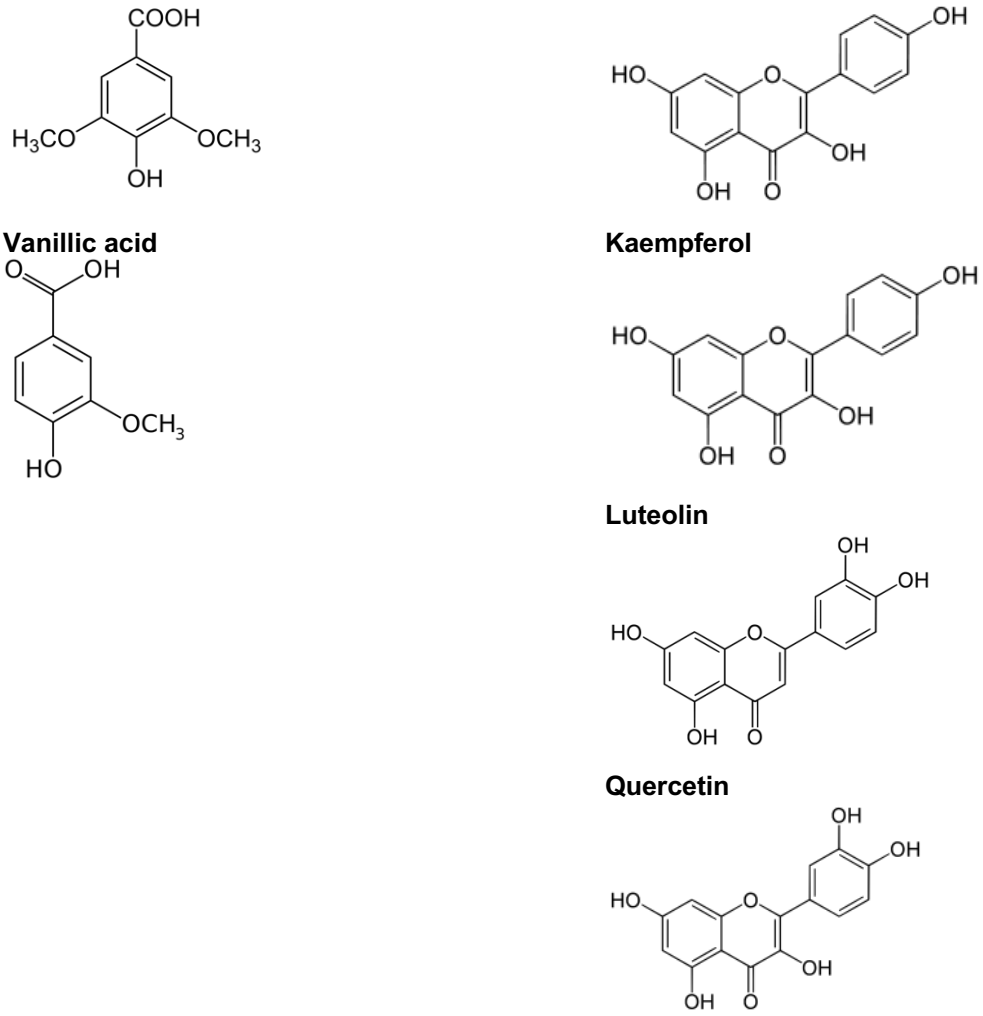


Figure 2.7.1. The chemical structure of the (a) phenolic acids and (b) flavonoids commonly found within honey. Depicting the hydroxycinnamic acid structure or hydroxybenzoic acid structure of the phenolic compounds. Adapted from Cianciosi *et. al.*[52]

These phenolic compounds play a role in the anti-inflammatory activity of honey by suppressing the pro-inflammatory effects of COX-2 and inducible nitric oxide (iNOS)[111] subsequently increasing NO end product's and decreasing prostaglandins.[114] Decreasing prostaglandin levels reduces oedema at the wound site and activates lymphocytes to produce antibodies.[116] The increased lymphocytic and phagocytic activity stimulates B and T cells, neutrophils, phagocytes, and monocytes subsequently secreting TNA-α, IL-1β and IL-6.[111,116] TNA-α is a growth factor at enhances angiogenesis and proliferation of fibroblasts and epithelial cells.[114] Furthermore, the production of antibodies increases NO subsequently increasing humoral immunity, promotes angiogenesis and antimicrobial activities.[116] The antibacterial effect of honey is partially attributed to its flavonoid content having the ability to inhibit bacterial RNA polymerase and degrade the bacterial cell membrane, and its phenolic

acid content playing a role in the destruction the bacterial cell wall causing cell leakage and death.[127]

Moreover, correlation has been made between honey colour, phenolic content, and antioxidant activity. This relationship is proportional indicating the darker the honey the higher the phenolic content, subsequently, the higher the antioxidant activity and vice versa. The polyphenols, carotenoids, and the caramelization of reducing sugars are responsible for honey colour.[128] This chemical caramelization is known as the Maillard reaction described as the nonenzymatic browning reaction through chemical interaction between free amino acids and reducing sugars resulting in Maillard Reaction Products (MRPs).[129] MRPs have been linked to antioxidant activity,[130] therefore, the combined antioxidant activity from both the phenolic compounds and MRPs can be estimated from the colour intensity of honey products.[128] However, honey can turn a lighter colour following crystallisation, this results from the formation of monohydrate glucose crystals (white in colour) that vary in number, quality, dimension, and shape.[108] This variation is due to honeys composition, specifically the ratio of water to glucose content, crystallisation occurs rapidly in honeys with a low water content and high glucose content.[108]

2.8 Honey protein and associated antimicrobial activity

Honey contains proteinaceous components consisting of proteins, peptides, enzymes, and free amino acids.[102] These proteins found in honey are of plant origin derived from the floral nectar and pollen suspended within the honey, or of animal origin, from the hypopharyngeal and mandibular glands of the bees.[131] Listed in Table 2.8.1 are several identified proteins, peptides, and enzymes.[7]

Table 2.8.1. The proteins including peptides and enzymes identified in several honeys by proteomic studies.

Identified Proteins	Reference
Major royal jelly protein (MRJP) 1	Won *et. al*.[132]
MRJPs 1−5, alpha-glucosidase, GOx, profilin, superoxide dismutase, short-chain dehydrogenase/reductase, apisimin and serine proteases	Rossano *et. al*.[133]
MRJPs 1-5, alpha-glucosidase, and bee defensin-1 (DF-1)	Di Girolamo *et. al*.[134]
MRJPs 1,2,5 and 7	Chua *et. al*.[135]
MRJPs 1-9, alpha-glucosidase, alpha-amylase, and GOx	Zhang *et. al*.[136]
MRJPs 1-9, alpha-glucosidase, alpha-amylase, GOx, transferrin 1, glucosylceramidase-like protein, and putative glucosylceramidase 4	Barutinskaite *et. al*.[137]

Erban *et. al.*[7] undertook a comprehensive mass spectrometry analysis of the protein content in several honeys, including the molecular mass profiles of the most abundant proteins.[7]

Table 2.8.2. The most abundant and molecular mass of proteins found within several honey sources as identified by *Erban et. al.*[7]

Identified Protein:	Molecular Mass (KDa):
DF-1	8.748
Elongation factor 1-α	9.627
Chymotrypsin inhibitor-like	14.639
Ferritin heavy polypeptide-like 17	30.321
Venom serine protease Bi-VSD	39.483
Chymotrypsin inhibitor	33.328; 7.325
Venom serine protease 34	44.438
Ubiquitin-60S ribosomal protein	52.076
Carboxypeptidase Q	52.947
Hsc 70-3/Hsc 70AB	56.12
GOx	67.937
Glucose dehydrogenase	70.123
Serine protease inhibitor 88Ea	88.45
Actin	137.51
Putative glucosylceramidase 4	155.27
Glucosylceramidase-like	158.51
Icarapi	187.41
Lactase-5	224.21
MRJP 9	230.25
MRJP 1	323.31
MRJP 3	323.31
MRJP 6	323.31
MRJP 7	323.31
Esterase B1	323.31
Lipase member H-A	323.31
Xanthine dehydrogenase	323.31
Hexamerin 110	323.31
MRJP 2	323.31; 22.892
Alpha-glucosidase III	41.199; 323.31
α-amylase	148.62; 323.31
MRJP 4	175; 323.31
MRJP 5	24.5; 323.31; 175.19

The presence of DF-1, and jelleins 1, 2, and 4 in honey are reported to have an antimicrobial effect through the stimulation of bacteriolysis.[7] DF-1 is an antimicrobial peptide found in the haemolymph and hypopharyngeal glands of the bee,[137] therefore the amount of DF-1 within honey differs due to variation in the production rate from individual bees.[138] These peptides function in the innate immune response of the bee, eliciting an antimicrobial effect against fungi, yeast, protozoa, Gram-positive and Gram-negative bacteria. DF-1 has an antimicrobial function by creating a pore or breakage in the cell membrane, disrupting membrane permeability, resulting in cell death.[118] This antimicrobial activity can be attributed to the

cationic character of the protein structure interacting with the negatively charged membranes of invading microorganisms.[139] Further reports suggest DF-1's role in wound healing is not only antimicrobial but through simultaneous stimulation of MMP-9 secretion from keratinocytes.[140] MMPs enable the disassociation of keratinocytes from the basement membrane and facilitates their migration, more specifically, MMP-9 is involved in the degradation of the ECM proteins such as type III and IV collagens and elastin promoting granulation tissue remodelling and re-epithilialisation.[141]

Jelleins 1, 2, and 4 are cleavage products of MRJP 1,[142] therefore, ubiquitous identification of MRJP 1, found in Table 2.8.1, suggests that jelleins 1, 2, and 4 are key role players in the antimicrobial properties of honey.[7] Won *et. al.*[132] identified that MRJP 1 is a GP consisting of 3-5% of N-linked oligosaccharides of the total molecular weight.[132] A study conducted by Brudzynski and Sjaarda[143] identified two distinctive antimicrobial functions of isolated GP samples containing MRJP 1 and associated jellein peptides these were; specific binding and agglutination of bacterial cells, and non-specific membrane permeabilization of bacterial cells.[143] Figure 2.8.1 depicts a scanning electron micrograph of morphological changes and subsequent cell death of *E. coli* and *Bacillus subtilis* post exposure to isolated GP samples containing MRJP 1 and associated jelleins 1, 2, and 4.[143]

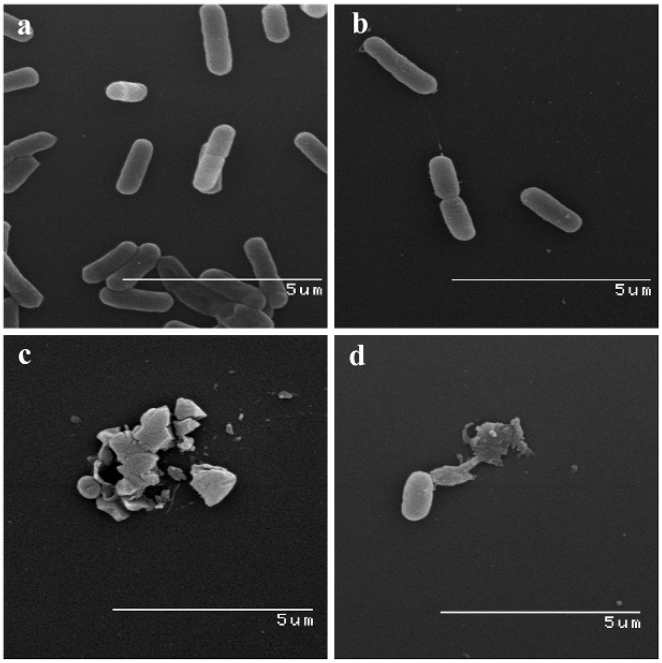


Figure 2.8.1. Scanning electron micrograph of GP-induced morphological changes and subsequent cell death of E. coli and B. subtilis in lag phase of the growth cycle. (a) Control culture of E. coli, (b) control culture of B. subtilis, (c) E. coli treated with bactericidal concentrations of MRJP-1 and Jellein containing GP sample, (d) B. subtilis treated with bactericidal concentrations of MRJP-1 and Jellein containing GP sample.[143]

Furthermore, MRJP-1 has an anti-inflammatory function by binding to the mannose receptors on human phagocytic cells, thereby inhibiting phagocytosis during inflammatory response. Other MRJP's, such as MRJP 3, have anti-inflammatory function by supressing the release of IL-2, IL-4, and interferon-γ.[142]

Antibacterial activity is also associated with GOx, this enzyme catalyses the oxidation of glucose and H_2O to gluconic acid and H_2O_2 as seen in 2.6.1.[129,144] H_2O_2 has been described as an oxidative biocide,[144] sterilizing the wound by eliciting oxidative damage to microorganisms through interaction with the bacterial cell wall, intracellular lipids, proteins, and nucleic acids.[109] This interaction removes electrons from the bacterial structures synthesizing hydroxyl radicals (Fenton-like reaction) that irreversibly damages DNA and inhibits microbial growth. This activity is increased following the dilution of honey associated with high GOx activity. With dilution, the continuous synthesis of H_2O_2 occurs as GOx can bind more readily to its substrate's glucose and H_2O.[144] H_2O_2 further elicits the production of VEGF aiding in the growth of new tissue at the site of the wound as mentioned previously.[1] The produced H_2O_2 is hydrolysed by catalase to H_2O and O_2, ensuring the efficient removal of H_2O_2 driving the reaction in a unilateral direction and prevents the oxidation of GOx.[117]

In addition, serine protease and protease inhibitors derived from bee venom are proteolytic enzymes that catalyse the degradation of proteins, hydrolysing the peptide bonds that link amino acids in the polypeptide chains of various proteins. Common proteases within honey include elastase, trypsin and chymotrypsin.[133] These enzymes are used by the bee as a defence mechanism eliciting immediate hypersensitivity reactions that can lead to anaphylaxis and, in severe cases, death.[145] Serine proteases play a role in remodelling the ECM, regulating inflammatory responses, growth factors, and cytokine signalling.[146] Serine protease activity is essential for cutaneous wound healing as these proteases play a role in the degradation of the collagen compounds found in the ECM, ensuring efficient removal of unwanted matrix during tissue remodelling.[147] It is when the balance between ECM degradation and tissue deposition is disrupted that chronic wounds are formed. Apart of maintaining equilibrium is the production/activation of serine proteases and the respective inhibitors.[148] Serine protease inhibitors, in general, irreversibly bind to the catalytic domains of serine proteases thereby inhibiting local and transient reactions in response to physiological or pathological cues.[149] Serine protease inhibitors play a role in protecting target proteins from degradation, blood coagulation, ion channel activity, activation of the complement system, fibrinolysis, and inflammation within multiple species.[150,151]

2.9 South African honey research

Previous research has been conducted on South African honeys, Serem and Bester[152] conducted research on six South African honeys from several botanical origins collected from the Western Cape, Eastern Cape, Southeast Mozambique, Agricultural Eucalyptus, Agricultural Orange, and Agricultural Litchi. This study analysed the physiochemical properties, antioxidant activity and *in vitro* protective effects, and conclusively identified honeys with greater catalase activity and/or darker in colour exhibited high concentrations of polyphenols and flavonoids, and antioxidant activity subsequently exhibited a high degree of cellular protection.[152] Furthermore, identifying a positive correlation between colour, polyphenol and flavonoid content, and antioxidant activity.[152]

A study conducted by Basson and Grobler[153] investigated the antimicrobial activity of four honey samples, two of which were foraged from local plant species these include *Erica* spp. (Fynbos honey) and *Leucospermum cordifolium* (Pincushion honey) whereas the other samples were produced from alien plant species from either Australia or New Zealand these include *Eucalyptus cladocalyx* (Bluegum honey) and *L. scoparium* (Manuka honey) respectively.[153] This study was conducted using several Gram-negative and - positive bacterial strains, concluding that the antimicrobial activity (minimum inhibitory concentrations (MICs)) of honeys produced from endemic plant species from South Africa did not differ to that of the alien plant species, demonstrating no significant increase in antimicrobial activity.[153]

Furthermore, Magoshi *et. al.*[154] undertook research on six South African Fynbos honeys foraged in the Fynbos biome of the Western Cape, this study aimed to determine the effects of each digestive phase on the associated antioxidant activity and nitric oxide induced immunomodulatory roles of each Fynbos honey in comparison to a Manuka honey control (UMF 15+).[154] This study demonstrated that both undigested and gastric-digested Fynbos honey had antioxidant properties potentially reducing the risk for cancer post gastroduodenal digestion.[154] Interestingly, this study further identified a loss in anti-inflammatory activity of the Manuka control post-digestion, in contrast the Fynbos honey maintained its anti-inflammatory activity following digestion.[154]

2.10 Apicultural influence and potential limitations of honey

Honey is a natural product, and its composition varies significantly depending on floral source, geographic location, and processing methods.[155] The protein, polyphenol, flavonoid, and sugar constituents within honey differ in concentration in different honey samples. These differences, with specific focus on the proteinaceous concentrations, can be explained based on the

different floral and apicultural origins of honey.[156] Forager honeybees are dependent on diverse forage, either monofloral or multifloral, to adequately meet their nutritional needs.[157] The large majority of the honey proteome consists of proteins secreted from the salivary and hypopharyngeal glands of forager bees. Nitrogen sources and other basic elements of protein synthesis need to be actively foraged by the bee for synthesis of bee-derived proteins. Sources of such elements include nectar and pollen collected by the bees from different origins, with resultant variation in the nutritional composition of the honey proteome.[156] Pollen is the main amino acid source for bees to facilitate gland development, hence, pollens of different plants are reported to act synergistically to influence host nutrition and resultant honey proteome.[156,157] Therefore, for the purpose of the study fresh honey samples will be used in a triplicate sample harvest to investigate the risk of variance within the honey proteome.

Additionally, this variability in honey components affects honeys therapeutic efficacy and consistency.[84,85] It is evident in most medicinal honey research that variations in honey composition are not acknowledged or investigated making comparisons between different studies a challenge.[84,85] With the lack of standardized methods to quantify active compounds, such as methylglyoxal (MGO) and hydrogen peroxide, consistent processing methods are used to mitigate large variations amongst medicinal honey sources.[84,85] Medicinal honey products are made from pooled, filtered, and sterilised honey batches. The pooling and collection from the same honey source limits batch-to-batch differences due to variances in the apicultural origin. Filtration of the honey removes any wax, dirt, and pollen particles as a preventative method against possible allergic reactions. Further sterilisation is done using γ-irradiation to destroy live bacteria or spores before use as a wound healing product.[1] Adherence to the above regulations, allows effective and safe products intended for use in wound applications.[158]

Moreover, honey products are prone to adulteration by beekeepers and honey producers especially when production is limited, and supply cannot meet the increasing demand.[159] Adulteration (addition of water, sugar, molasses, and prohibited antibiotics), premature harvesting, and sugar supplementing during nectar periods increases the yield of honey.[159,160] Furthermore, honey quality can be diminished through repeated heating for recrystallisation, pasteurisation, and packing of the product, the sale of lower quality imported products mixed with domestic honeys, and adding invert sugar for increase commercial weight.[159,160]

Therefore, international standards for honey were developed and approved by the Commission of Food Code from 2001 (Codex Alimentarius: CODEX STAN12-1981 revision

2001) and the European Directive for honey.[159,160] Honey quality according to these standards is determined using its organoleptic characteristics, differentiating dominant pollens, and physiochemical properties.[160] International standards require quantitative analysis of honey composition ensuring compliance with the following guidelines: sucrose content ≤ 5%, fructose 31–42%, glucose 23–32%, reducing sugar ≥ 60%, moisture content ≤ 21%, water-insoluble content ≤ 0.1%, conductivity ≤ 0.8 mS/cm, ash content ≤ 0.6%, diastase activity ≥ 8 DN, and hydroxymethylfurfural content ≤ 40 mg/kg.[159] For the purposes of this study, the investigation of quantitative analysis of several international standards will be investigate to mitigate the risk of reporting on adulterated honey products.

Another point of concern when researching honey is the possibility of increased adverse events due to patient allergies.[161,162] As majority of the protein content within honey is derived from the bee, the pollen proteins and glandular proteins are hypothesised to be the source of honey allergy in patients. Although rare, the incidence of honey allergies in the general population is not clearly know, it is estimated to be <0.001%.[163] A study conducted by Aguiar *et. al.*[164] researched the effects of honey on a 40-year-old female with a suspected allergy to honey.[164] 9 types of different honey sources were introduced to the patient through a prick-to-prick test, 30 minutes following the administration the patient experienced anaphylactic symptoms for which she was treated apporpriatly.[164] Interestingly the patient was exposed to royal jelly extract and propolis where no symptoms of anaphylaxis were observed.[164] Additionally honey allergies have been reported Karakaya and Kalyoncu,[165] Kalyoncu,[166] and Cifuentes.[167] However, due to low incidences of honey allergies in patients, research of medicinal honey products and associated anaphylaxis is very limited therefore adding to the limitations of honey research.[164–167] Increasing research on various honey sources and isolated honey components, like the proteins, may aid in improving our understanding of allergies to medicinal honeys and associated bioactive compounds.

2.11 Project rationale

The introduction of antibiotics in the 1940's made honey a less favourable method of treatment,[1] however, with recent increases in the prevalence of antibiotic resistant bacterial infections (MRSA),[168,169] as well as *in vitro* and *in vivo* evidence supporting honey's natural broad spectrum antimicrobial properties, honey has become a more viable option in clinical medicine.[1] Additionally, an investigation into the honey proteome for its role in wound healing is motivated by honey's diverse therapeutic benefits, including antimicrobial, anti-inflammatory, antioxidant, and tissue-regenerative effects.[170] These wound healing properties originate from honey's complex composition, which consists of proteins, phenolic compounds, and other bioactive molecules.[170] Analysis of the honey proteome can aid in identification of

the role the proteins and play in elucidating these therapeutic effect, potentially enabling the development of more targeted and efficient wound care treatments.[170]

Medical grade honey treatments are imported from New Zealand and have a high associated cost. [171]Imported medicinal products are subject to additional international shipping costs and import tariffs that do not apply to locally sourced products. Locally produced honey, especially those with observed antimicrobial properties, are more cost-effective. For instance, the average cost of honey treatment was reported at R0.49 per patient, compared to R12.03 for IntraSite Gel treatment in 2006.[172] It is important to further acknowledge the relationship between African culture and natural medicines. Herbal medicine is rooted in the cultural beliefs and practices of African communities, where illness is often perceived as having both natural and supernatural causes.[173] African medicine products and medicines are viewed as natural and safer compared to conventional Western therapies, making it a preferred choice for many in sub-Saharan Africa due to its affordability and accessibility.[173]

The unique biodiversity and the species of bee, *A. mellifera scutellate*, found in the Fynbos biosphere in the Cape Floristic Kingdom of South Africa provides a unique honey type whose composition includes high levels of polyphenols and flavonoids that is maintained after simulated digestion, unlike Manuka honey which experiences a loss in antioxidant capacity post-digestion.[154] In an endeavour to further develop this honey as a medical grade honey it is necessary to determine the composition and the bioactivity of the constituent fractions, including the protein fraction to determine the contribution of this fraction to bioactivity and its potential contribution to wound healing using relevant *in vitro* models.

It is evident that most honey research is directed at the anti-inflammatory, antimicrobial, and antioxidant functions of the sugars, glyoxal derivatives, and phenolic compounds whilst the proteins are largely overlooked. The protein content of honey is measured between 0.4-0.5%, making the protein and peptide extraction a difficult objective to achieve.[7] However difficult, extraction has been achieved by Brudzynski and Sjaarda,[143] Won *et. al*.,[132] Rossano *et. al*.,[133] Di Girolamo *et. al*.,[134] Chua *et. al*.,[135] Zhang *et. al*.,[136] and Borutinskaite *et. al*.[137] This study will be amongst the few honey proteome studies that gives insight into the anti-inflammatory, antibacterial, antioxidant, and mitogenic properties of the honey proteome.

2.12 Aims

Therefore, the aim of this study was to determine the bioactivity and mitogenic properties of whole Fynbos honey and their isolated protein fractions.

2.13 Objectives

The aims were achieved through the following objectives:

1. To optimise gel filtration chromatography conditions to efficiently isolate the protein fraction of honey.
2. To determine the physiochemical properties of the honey samples and protein fractions.
3. To identify the most abundant proteins, and to determine the molecular masses using SDS-PAGE assay.
4. To determine the cytotoxicity and cell migratory effects of both the honey and protein extracts on human keratinocytes (HaCaT) and murine fibroblasts (SC-1) using the SRB assay and the scratch migration assay.
5. To determine, with the TEAC, ORAC and CAA assay, the chemical and cellular antioxidant activity of each honey and their respective protein fractions.
6. To determine the anti-inflammatory activity of each honey and their respective protein fractions using the LPS-induced NO/RAW 264.7 cell model.
7. To determine the minimum inhibitory concentration (MIC) of the honey and the isolated protein extracts against *S. aureus* and *E. coli* using the microbroth dilution assay.

Chapter 3: The protein isolation and physiochemical properties of selected South African honeys and protein extracts

3.1 Introduction

The universally accepted definition of quality, according to international standards for quality management is “the degree to which a set of inherent properties meets the requirements” is the universal definition of quality as an international standard for quality management.[160] The international standards for honey are monitored by through the Codex Alimentarius: CODEX STAN12-1981 revision 2001) and the European Directive for honey.[159,160] Honey quality according to these standards is determined using its organoleptic characteristics, differentiating dominant pollens, and physiochemical properties.[160]

Proteins and amino acids in honey account for 0.1-0.5% of its composition, and their isolation is typically achieved through dialysis against water followed by lyophilisation.[174] During dialysis, small unwanted molecules such as the sugars, polyphenols, H_2O_2 and MGO, are selectively removed by passive diffusion, while larger macromolecules like peptides and proteins are retained. Repeated changes of the dialysate buffer reduces the concentration of the smaller contaminating molecules.[175] Alternatively, gel filtration chromatography uses the principle of size exclusion to separate small molecules, such as sugars and polyphenols, from larger peptides and proteins more selectively.[176] Compared to dialysis, gel filtration chromatography offers advantages in optimising flow rates and elution volumes , minimising dilution effects, and reducing isolation time.[174] Additionally, it lowers costs by decreasing reliance on expensive lyophilisation equipment, shortening processing time, and mitigating dilution effects caused by increased dialysate volume.[174]

Following isolation, spot testing enables the identification of different molecules in the collected fractions. In honey analysis, this involves identifying the sugar and protein fractions using assays like the Benedict’s assay for reducing sugars[177] and the bicinchoninic acid (BCA) assay[178] for proteins. However both methods rely on the reduction of Cu^{2+} ions to Cu^{1+}, either through the interaction of peptide bonds in proteins with Cu^{2+} forming a Cu^{1+}-protein complex or the interaction of Cu^{2+} with the free carbonyl groups of reducing sugars.[177,178] Notably, polyphenols can interfere in this reaction necessitating careful selection of assays.[179] In this study, the Bradfords was used for protein determination, while the Seliwanoff’s assay was employed sugar identification and quantification. Further characterisation of honey proteins was performed using gel electrophoresis, such as SDS-PAGE, which allows for the tentative

identification of specific proteins based on their relative molecular masses, providing a more detailed characterisation of honey composition.

Beyond proteins and sugars, honey contains phenolic compounds, which are classified into simple phenols and polyphenols, synthesised by plants. Both older and recent publications have used colorimetric absorbance methods to correlate polyphenol content with antioxidant activity.[174] The total polyphenol content (TPC) assay oxidises phenolic compounds present in honey through interaction with Folin–Ciocalteu reagent, reducing phosphomolybdic acid and phosphotungstic acid to molybdenum and tungsten oxides, producing a blue colour. The colour intensity is directly proportional to the phenolic content in the sample.[174] To confirm the presence of polyphenols the TPC assay is often coupled with colour absorbance, further validating the presence or absence of these compounds and their potential antioxidant activity.[174]

By integrating physicochemical characterization techniques with biochemical assays, a comprehensive evaluation of honey quality can be achieved. The precise identification and quantification of proteins, sugars, and polyphenols contribute to a deeper understanding of honey composition and its adherence to international quality standards.

The aim of the research undertaken in this chapter was to isolate the protein fraction of selected South African honey samples and characterise their physiochemical properties.

The specific objectives for this chapter were:

1. To optimise gel filtration chromatography conditions to efficiently isolate the protein fraction of honey.
2. To determine the physiochemical properties of the honey samples and protein fractions.
3. To identify the most abundant proteins, and to determine the molecular masses using SDS-PAGE assay.

3.2 Materials

Honey samples

Eight honey samples were collected between 2020-2022 from established bee keepers, and /or rural farm stalls, and/or international medicinal stores. Established bee keepers and rural farm stalls located in the Western Cape area of South Africa were of particular interest as these bees would most likeley forage in the biome of interest for this study, the Fynbos biome.

International medicinal stored such as those located in New Zealand and the United States of America (USA) were used to collect the control honeys to ensure the integrity of the natural compostion of these medical grade control samples. These samples include medical grade Manuka (MA) (control, n=1) from New Zealand, Buckwheat (BU) (control, n=1) from the USA, multifloral (MF) (n=1), and Fynbos honey (FB) (n=5) from South Africa. These n values refer to jars of honey from a single source. To increase reproducability of results triplicate samples from each jar were extracted and used as individual samples in experimentation.

Reagents, equipment and disposable plasticware

Reagents included: fluorescent treponemal antibody (FTA) hemagglutination buffer 0.1 M and 0.01 M phosphate buffered saline (PBS), fructose, Coomassie Blue G250, Sephadex G-25, 85% phosphoric acid (H_3PO_4), 97% sulfuric acid (H_2SO_4) (Merck Chemicals, Modderfontein, SA), 99,7% acetic acid, 99,6% methanol, D-sorbitol, xylenol orange, 97% H_2SO_4, ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) (Sigma Aldrich, Atlasville, SA), 10X Tris Glycine SDS running buffer (Thermo Fisher Scientific, Johannesburg, SA), Precision Plus Protein™ unstained protein standard mixture (BioRad, Lasec, Western Cape, SA). Reverse osmosis and deionised H_2O (ddH_2O) was prepared inhouse using an Elga Genetics water purification system.

Equipment included: 132A 40L water bath, Hermle Z300 centrifuge (Hermle LaborTechnik GmbH, Wehingen, Germany), Nuaire incubator, 105 magnetic stirrer with heating (Labotech, Gauteng, SA), Synergy II fluorescent plate reader (Biotek Instruments, Inc., Winooski, USA), Omega FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany), Pharmacia Fine Chemicals column (12.5 x 500 mm), Gilson Minipuls 2 peristaltic pump, LKB Bromma 2212 Helirac fraction collector, Hermle Z 100 M mini centrifuge (Hermle LaborTechnik GmbH, Wehingen, Germany), electrophoresis power supply EPS 301 (Amersham Pharmacia Biotech, Amersham, United Kingdom (UK)), Invitrogen XCell SureLock™ Novex mini cell tank (Thermo Fisher Scientific, Johannesburg, SA), Gel Doc™ EZ Imager (Lasec, BioRad, Western Cape, SA), Labconco Lyophiliser (Vacutec, Roodepoort, SA), FA2004 scale, Precisa XT 120A (Lasec, Gauteng, SA), ImageLab (BioRad, Lasec, Western Cape, SA), and Fijji/ImageJ software (softonics),

Disposable plasticware included: Novex value 4-20% Tris-Glycine gels (1.0 mm x 10 well) (Thermo Fisher Scientific, Johannesburg, SA), polystyrene 96 well plates, 50 mL/15 mL Falcon tubes, Eppendorf tubes, 0.45 uM Millipore syringe filters, 20 mL syringes, 0,1-10 uL,

20-200 uL, 100-1000 uL pipette tips which were obtained from Greiner Bio-one supplied by Lasec, Johannesburg, SA.

Laboratory facilities

All research was conducted in the research laboratories associated to the Departments of Anatomy and Pharmacology of the Faculty of Health Sciences at the University of Pretoria, SA.

3.3 Methods

Sample preparation

All honey samples were prepared to an initial 50% (v/v) with ddH_2O and were then further diluted to a 25% (v/v) solution. The samples were centrifuged at 2000 x *g* for 10 minutes and filtered using Whatman 541 filter paper and 0.45 µm Millipore syringe filters. All samples were stored in the -80°C freezer protected from the light.

3.3.1 Protein isolation from whole honey samples

Sephadex G-25 is a gel-filtration soft bead-form desalting gel prepared by cross-linking dextran with epichlorohydrin, used as the stationary phase to separate the di- and mono-saccharides from the protein content.[176]

Gel filtration chromatography separates molecules by size as they pass through a column packed with porous beads, where larger molecules elute faster as they are not able to pass through selective porous beads.[180] In contrast smaller molecules elute later as they are absorbed and passed through the porous beads.[180] Separation occurs as these differently sized molecules pass through the column's stationary phase at different speeds.[180] Using gel filtration chromatography the different fractions were collected and the protein and sugar content of the fractions was determined with the Coomassie Blue and the Seliwanoff's spot tests.

Protein isolation

Gel filtration chromatography was used to isolate the protein content of 25% (v/v) honey samples. Molecules are partitioned between a mobile phase and a stationary phase due to the relative sizes of the molecules within the complex sample mixture.[176] Protein was isolated from the whole honey samples in triplicate and analysed separately. The method used was adapted from Ó'Fágáin *et. al*.[176] A 0.1 M PBS solution was prepared by dissolving 9.3 g of FTA hemagglutination buffer (7.65 g/L NaCl, 1268.8 mg/L, Na_2HPO_4, 0.1 g/L NaH_2PO_4, 211.3

mg/L KH_2PO_4, pH 7.2) in 1 L of ddH_2O and diluted 10x to a 0.01 M solution. Sephadex G-25 powder, approximately 30 g, was equilibrated in 150 mL of 0.1 M PBS and stored for ± 16 h at 20°C. A Pharmacia Fine Chemicals gel chromatography column (12.5 x 500 mm) was tightly packed with the swollen Sephadex G-25 gel and connected to a Gilson Minipuls 2 peristaltic pump, set at a flow rate of 350 µL/min. The packed column was washed with 60 mL of ddH_2O prior to the introduction of 8 mL of each 25% (v/v) honey sample. The samples were then eluted using 250 mL of 0.01 M PBS (mobile phase) pumped at a constant flowrate of 350 µL/min. Eluted fractions were collected at 4-minute intervals in glass test tubes using a LKB Bromma 2212 Helirac fraction collector. The collected fractions were stored at -80°C in the dark.

Coomassie Blue G-250 spot test

Coomassie Blue exists in two colour isoforms, red and blue,[181] the red isoform is protonated whilst the blue form is unprotonated.[182] When the acidic Coomassie blue solution binds to protein the positive charges prevent protonation therefore converting the stain solution to the blue isoform that then can be quantified.[182]

The Coomassie blue G-250 staining solution was prepared by dissolving 0.2 g of Coomassie Blue G-250 in 100 mL of 70% ethanol and further diluted with 1.8 L of ddH_2O, before 42 g of citric acid was then added to the Coomassie Blue G-250 solution with vigorous mixing. An aliquot of 100 µL of each collected fraction was placed on 12 well ceramic plates and left in the fume hood to evaporate for ± 16 h. The dried fractions were dissolved in 100 µL of ddH_2O and then 50 µL Coomassie Blue G-250 diluted in 10% H_3PO_4 was added. Each fraction was further diluted with 100 uL of ddH_2O to allow for colour development to show presence of proteins. Protein positive fractions presented as a bright blue colour; these fractions were pooled, aliquoted into 2 mL Eppendorf tubes, and stored in the dark at -80°C. No further sample processing was undertaken, and the pooled samples, were used as such for further evaluation for bioactivity as described in Chapters 4 - 6.

Sugar spot test

The Seliwanoff's assay is a colour reaction for ketose sugars, based on the rapid dehydration of ketoses in comparison to aldose sugars. Further condensation with resorcinol in an acidic environment gives a bright red colour in the presence of ketose sugars.[183]

The method used in this study was adapted from Monsigny *et. al*.[184] where 20 µL aliquots of each collected fraction was placed on 12 well ceramic plates and the 20 µL of 6 mg/mL

resorcinol and 200 µL of 75% H_2SO_4 was added. After heating to 90°C for 15 minutes, 100 µL aliquots were diluted in 100 µL, 0.01 M PBS. The presence of sugars was indicated by the development a bright red to dark maroon colour. Photographs were taken to identify sugar positive fractions and determine overlap with protein positive fractions.

3.3.2 Determination of physiochemical properties

Colour absorbance

High absorbance readings in honey samples indicate darker honeys, suggesting a high MRP and polyphenol content.[48] A strong absorbance reading at 560-720 nm is due to the presence of MRPs and at 420-450 nm is due to polyphenols.[185] The absorbance is defined as the difference between 450 nm and 720 nm, the net difference between the two readings was calculated as follows: $A_{450} - A_{720}$ to determine an overall absorbance value for each sample. A volume of 300 µL of each honey sample at 25% (v/v) in 0.01 M PBS was added to a 96 well plate. A 300 µL volume of 0.01M PBS was used as a blank. The absorbance was measured using the Omega FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). Results were reported as A_{450} in absorbance units (AU) and colour (A_{450}-$A7_{20}$) in AU.

Bradford assay

In the Bradford assay, the Coomassie Blue G-250 stain, forms a protein-dye complex. The protein-dye complex has a high extinction coefficient thus leading to high sensitivity in protein concentration measurement. The protein-dye complex has a high extinction coefficient thus leading to high sensitivity in protein concentration measurement. The binding of the dye to protein is a very rapid process (approximately 2 min), and the protein-dye complex remains dispersed in solution for a relatively long time (approximately 1 h), thus making the procedure very rapid and yet not requiring critical timing for the assay.[181] The method used was adapted from the method outlined by Bradford, (1976).[181]

For the preparation of a standard curve, using a 50 µg/mL stock bovine serum albumin (BSA) solution, a concentration series of 0 - 50 µg/mL in 100 µL was prepared. 50 mL of a 5X Coomassie G-250 solution diluted with ddH_2O was prepared. A 25 µL volume of the BSA standard, 25% honey or the protein fractions pipetted in triplicate in separate wells in a 96 well plate. To each sample 105 µL of the 5X Coomassie G-250 solution was added. The absorbance was measured at 595 nm using the Synergy II (Biotek Instruments, Inc., Winooski, USA). The BSA standard curve (r^2>0.95) was used to determine the protein content in the

honey and protein fraction. The concentrations were expressed as mg/g, after the weight of the honey samples and protein fractions was taken into account.

Seliwanoffs sugar determination assay

The Seliwanoff's reaction as described above was adapted to quantify sugar content, the same principle applies with the 96-well plate assay. This method was adapted from Monsigny *et. al*.[184] Stock solutions of 10 mg/mL fructose were diluted to prepare a concentration series of 0 - 200 µg/mL. To 80 µL of the fructose, 25% (v/v) honey, or protein fractions 80 µL, 6 mg/mL resorcinol and 800 µL, 75% H_2SO_4 was added. Once mixed, the samples were heated in boiling ddH_2O for 1 minute. Aliquots of 140 µL were transferred to the wells of a 96 well plate and then the absorbance was measured at 480 nm using the Synergy II (Biotek Instruments, Inc., Winooski, USA). The fructose standard curve (r^2>0.95) was used to determine the sugar content of the honey and protein fractions, expressed as mg/100 g, after the weight of the honey samples and protein fractions was taken into account.

Total polyphenol content

In the total polyphenol content (TPC) assay the phenolic compounds are oxidised with Folin–Ciocalteu (F-C) reagent. In the process phosphomolybdic acid, and phosphotungstic acid, are reduced to molybdenum and tungsten to produce a blue colour. The colour intensity is directly proportional to the content of phenolic compounds present in the sample.[174] The TPC was determined using the F-C method as modified and described by Serem and Bester.[152]

A 0.2 mg/mL gallic acid stock solution was used to prepare a standard curve of 0 – 0.2 mg/mL. Working solutions of 10 mL of 15X F-C reagent and 10 mL of 7.5% anhydrous sodium carbonate (Na_2CO_3) solution were prepared. To 10 µL of the GA concentration series, 25% (v/v) honey solutions and protein fractions, 50 µL of 15X F-C reagent was added followed by 50 µL of 7.5% Na_2CO_3.The blanks were 10 µL ddH_2O or 0.01M PBS. The plate was incubated for 10 minutes at 30°C and absorbance was measured at 630 nm using the Synergy II (Biotek Instruments, Inc., Winooski, USA). The gallic acid standard curve (r^2>0.95) was used to determine the TPC expressed as µg gallic acid equivalents (GAE)/100g, after the weight of the honey samples and protein fractions was taken into account.

Molecular mass profiles using SDS-polyacrylamide gel electrophoresis

In Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated by their molecular weight determined through the migration through a gel matrix subjected to a direct current electrical field.[186]

SDS is an anionic detergent containing a long aliphatic chain and negatively charged sulphate groups giving the detergent a strong negative charge.[187] As the protein and SDS are boiled the heat disrupts the secondary and tertiary structures of the proteins where the SDS molecules bind in an almost fixed mass ratio to the hydrophobic residues of the protein through its alkyl chains, coating the polypeptide chains of the protein and overwhelming its charge.[187,188] Mercaptoethanol breaks any disulfide bonds between proteins resulting in individual negatively charged linear chains. The pores in the polyacrylamide gel is determined by the percentage gel and cross-links, and this selectively retards the migration of larger proteins when subjected to an electric field enabling the negatively charged proteins to migrate through the polyacrylamide gel towards the positively charged electrode.[186] With completion of a run, usually when the bromophenol blue (BPB) marker reaches the bottom of the gel, the proteins will have migrated according to size. The mass of the separated proteins can be determined relative to the molecular mass of a mixed mass protein standard run in a separate well of the gel.

For sample preparation, a 3X Laemmli buffer of 148 mg of Tris-HCl, and 0.3 g of SDS dissolved in 2 mL H_2O was prepared. Then 1 mL glycerol was added followed by 10 mg of bromophenol blue and then the solution was made to a final volume of 4.25 mL with ddH_2O. A volume of 750 µL of β-mercaptoethanol was added to the 3X Laemmli buffer right before use. To 50 µL of each honey sample and protein fraction, 25 µL of 3X Laemmli buffer was added. The samples were boiled for 10 minutes at 100°C before centrifugation 16000 x *g* for 5 minutes using a Hermle Z 100 M microcentrifuge.

A 20 µL volume of the samples and 10 µL of the Precision Plus Protein™ unstained standard (molecular mass range 10 - 250 kDa) were loaded into separate wells of the Novex value 4-20% Tris-glycine gels. The gels were run in the Invitrogen XCell SureLock™ Novex mini cell tank filled with 1X Tris glycine SDS running buffer (80 mL of 10X Tris glycine SDS running buffer in 720 mL of deionised water). The gels were run at 60 V and 55 mA for ± 20 minutes and then the voltage was increased to 200 V for ± 15 minutes. The gels are removed from the plastic casing and stained in colloidal Coomassie G-250 for 1 h and destained using ddH_2O for ± 16 h. Destained gels were imaged using the BioRad Gel Doc™ EZ Imager and analysed using the BioRad Image Lab software. The relative migration distance of the Precision Plus Protein™ standard bands and isolated protein fraction bands were measure using the linear measuring tool in ImageJ. The measurements were taken consistently from the bottom of the gel well to the middle of the detected protein bands relative to the bromophenol blue migration.

From the Precision Plus Protein™ standard a standard curve of log molecular mass vs Rf was constructed and then the tentative molecular mass of the proteins in the isolated protein fractions was determined through extrapolation from Table 2.8.2.

Data management and statistical analysis

Data reported is an average of three experiments where each measurement was in triplicate, subsequently, 9 data points were generated per sample (independent variable) treated as dependent variables. All data was expressed as mean ± standard error of mean (SEM) of the triplicate experiments analysed using Microsoft 365 Excel 2023. Determination of parametric and non-parametric data was conducted using the D'Agostino and Pearson test; and Shapiro-Wilk test using the GraphPad prism software version 9.5.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Data was statistically analysed using one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test was conducted for comparison of means using the same statistical software.

3.4 Results

3.4.1 Honey samples

This study is an exploratory study, as to our knowledge no prior investigation has been done on the bioactivity of the isolated protein content from South African Fynbos honey. This study serves a dual purpose: (a) to determine potential parallels in activity between Fynbos honey and medical grade Manuka honey (UMF 150+), and (b) to evaluate the bioactivity of the protein content within various honey products. Eight honey samples, listed in Table 3.4.1, were utilised for this investigation. Despite the relatively small sample size, this study will provide fundamental insights that will lay the groundwork for more comprehensive future research.

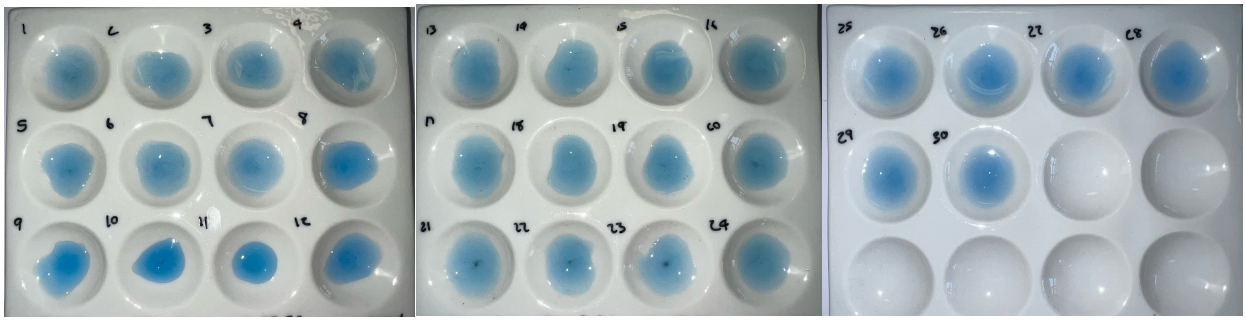
Table 3.4.1. The sample codes, floral type, and area of origin of the honeys used in this study.

Sample Code	Floral Type	Area of Origin
MA	Manuka (UMF 150+)	New Zealand
BU	Buckwheat	United States of America
FB1	Fynbos	Janwillemsfontein, Western Cape
FB2	Fynbos	Wolvefontein Farms, Gouritsmond, Western Cape
FB3	Canola and Fynbos	Stilbaai, Western Cape
FB6	Fynbos	Stilbaai, Western Cape
FB9	Fynbos	Stilbaai, Western Cape
MF1	Multifloral	Stilbaai, Western Cape

3.4.2 Protein isolation

The protein and sugar content of each fraction was determined with the Coomassie Blue G-250 and the Seliwanoff's spot tests Figure 3.4.1. The Coomassie Blue G-250 spot test indicates protein concentration based on colour intensity, where a light blue colour signifies a low protein concentration, while a dark blue colour represents a high protein concentration. Similarly, in the Seliwanoff spot test, a light orange colour corresponds to a low sugar content, whereas a dark orange or red colour indicates a high sugar content. The sugar fractions with a dark orange colour (Figure 3.4.1b) were 15-28 for MA, 16-29 for BU, 14-26 for MF1, and 14-29 for the FB extracts. Proteins staining blue (Figure 3.4.1a) was detected in all fractions, and only fractions with the highest protein content (indicated by a dark blue colour) and lowest sugar content (indicated by the light orange colour) were selected for further experimentation. These were fractions 8-13 for MA, 9-14 for BU, 8-13 for MF1, and 7-13 for the FB extracts. Figure 3.4.1 is a representative of the Coomassie Blue G-250 and the Seliwanoff's spot tests, only depicting one sample (Sample FB9) and its corresponding fractions.

a.



b.

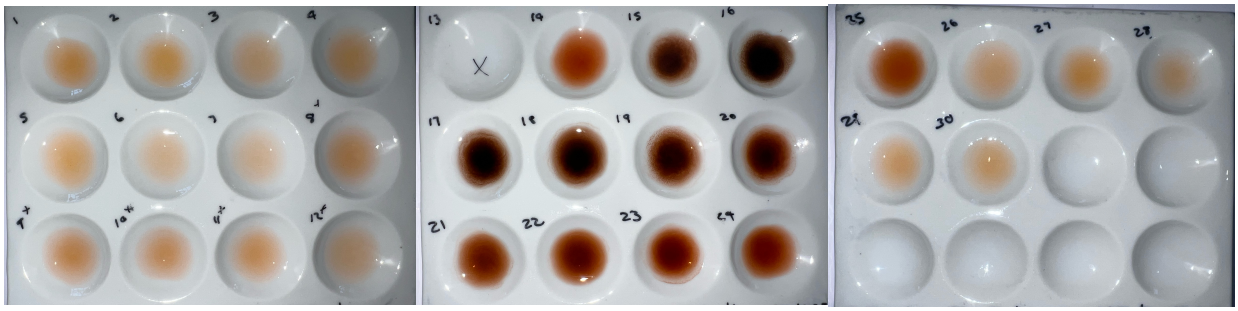


Figure 3.4.1. The detection of protein and sugar content in sample FB9, extraction 1, using (a) the Coomassie blue spot test and (b) the Seliwanoffs spot test, respectively, in the fractions following separation using gel filtration chromatography.

The protein and sugar content of each honey sample and protein fraction is provided in Table 3.4.2. According to this table the honey sample with the highest protein concentration was sample BU When the Fynbos honey samples were compared with MA and BU controls, honeys FB6 and FB9 were increased compared to MA and comparable to BU (Table 3.4.2). Manuka honey is widely used as a control in medicinal honey research due to its well-

documented antibacterial, antioxidant, and wound-healing properties, which are primarily attributed to its high methylglyoxal (MGO) content. By using Manuka honey as a benchmark, researchers can compare the bioactive properties of other medicinal honeys, helping to determine whether their therapeutic effects stem from similar or distinct mechanisms. Additionally, its standardized grading system, such as the Unique Manuka Factor (UMF), ensures consistency and reproducibility in studies, making it a reliable reference for evaluating the efficacy of alternative medicinal honeys.

Table 3.4.2. The repeats, the fractions, and protein and sugar content of each honey sample.

		Protein		Sugar	
Honey	**Repeat**	**Fractions**	**Protein (mg/g)**	**Fractions**	**Fructose (mg/100 g)**
MA	1	8-12	0.12 ± 0.01	15-25	16.33 ± 1.08
	2	8-13	0.12 ± 0.01	15-25	14.57 ± 1.93
	3	8-13	0.11 ± 0.01	15-28	19.32 ± 1.96
BU	1	9-14	0.14 ± 0.01^{b}	17-29	7.42 ± 0.58^{a}
	2	9-14	**0.15 ± 0.01**	17-28	13.00 ± 0.38
	3	9-14	**0.15 ± 0.01**^{cccc}	16-28	15.12 ± 0.19
MF1	1	8-13	0.03 ± 0.01^{aaaa}	15-26	7.30 ± 0.75^{aaa}
	2	8-12	0.06 ± 0.01^{bbbb}	14-25	16.35 ± 1.26
	3	8-13	0.06 ± 0.01^{cccc}	15-25	19.96 ± 1.24
FB1	1	8-12	0.05 ± 0.01^{aaaa}	14-25	9.66 ± 2.28
	2	8-11	0.053± 0.01^{bbbb}	14-25	15.91 ± 1.14
	3	9-13	0.05 ± 0.01^{cccc}	15-27	16.01 ± 1.59
FB2	1	8-11	0.04 ± 0.01^{aaaa}	14-23	14.16 ± 1.08
	2	8-11	0.05 ± 0.01^{bbbb}	14-22	21.80 ± 2.88
	3	8-12	0.11 ± 0.01	15-27	25.71 ± 1.66
FB3	1	8-12	0.04 ± 0.01^{aaaa}	14-23	9.87 ± 1.14
	2	8-12	0.05 ± 0.01^{bbbb}	15-22	23.58 ± 1.92^{b}
	3	8-12	0.05 ± 0.01^{cccc}	15-25	14.03 ± 0.86
FB6	1	9-12	**0.14 ± 0.01**^{a}	16-29	13.00 ± 3.49
	2	9-13	0.12 ± 0.01	16-25	29.59 ± 0.63^{bbbb}
	3	7-12	011 ± 0.01	14-23	23.15 ± 1.29
FB9	1	8-12	0.12 ± 0.01	14-25	17.41 ± 3.09
	2	8-13	0.13 ± 0.01	16-25	26.83 ± 1.81^{bbb}
	3	8-13	0.13 ± 0.01	15-24	24.14 ± 1.60

Bold= samples with the highest concentration in each extract group
Statistical significance to the MA control extract 1 ([a]), extract 2 ([b]), and extract 3 ([c]) is indicated as P<0.05[a] or [b] or [c]P<0.01[aa] or [bb] or [cc]P<0.001[aaa] or [bbb] or [ccc]P<0.0001[aaaa] or [bbbb] or [cccc]

3.4.3 Physiochemical properties

Using the Bradford method the protein content of the honeys was compared with the isolated protein fractions (Figure 3.4.2).

The average protein content for the honey samples was 0.95 ± 0.07 mg/g for MA, 1.33 ± 0.15 mg/g for BU and for MF1 this was 0.88 ± 0.11 mg/g. For the FB honey the highest concentration was 1.95 ± 0.01 mg/g for FB6 and the lowest of 0.72 ± 0.06 mg/g for FB3 (Figure 3.4.2). Compared with MA, the measured protein content was significantly increased for BU (p=<0.0001) and FB6 (1.19 ± 0.1 mg/g, p=<0.0001), whilst sample FB2 (0.78 ± 0.08 mg/g, p=<0.0001) and FB3 (0.72 ± 0.06, p=<0.0001) was significantly decreased.

The average protein concentration of the collected protein fractions were 0.12 ± 0.01 mg/g for MA, 0.15 ± 0.01 mg/g for BU and for MF1 was 0.05 ± 0.01 mg/g. For the FB honey the highest concentration was 0.13 ± 0.01 mg/g for FB6 and the lowest was 0.04 ± 0.01 mg/g for FB3 (Figure 3.4.2). For the protein fractions, compared with MA, the protein content of MF1(p=<0.0001), FB1 (0.05 ± 0.01 mg/g, p=<0.0001), FB2 (0.07 ± 0.02, p=<0.0001) and FB3 (0.04 ± 0.01 mg/g, p=<0.0001) was significantly lower, while the remaining samples had similar protein content.

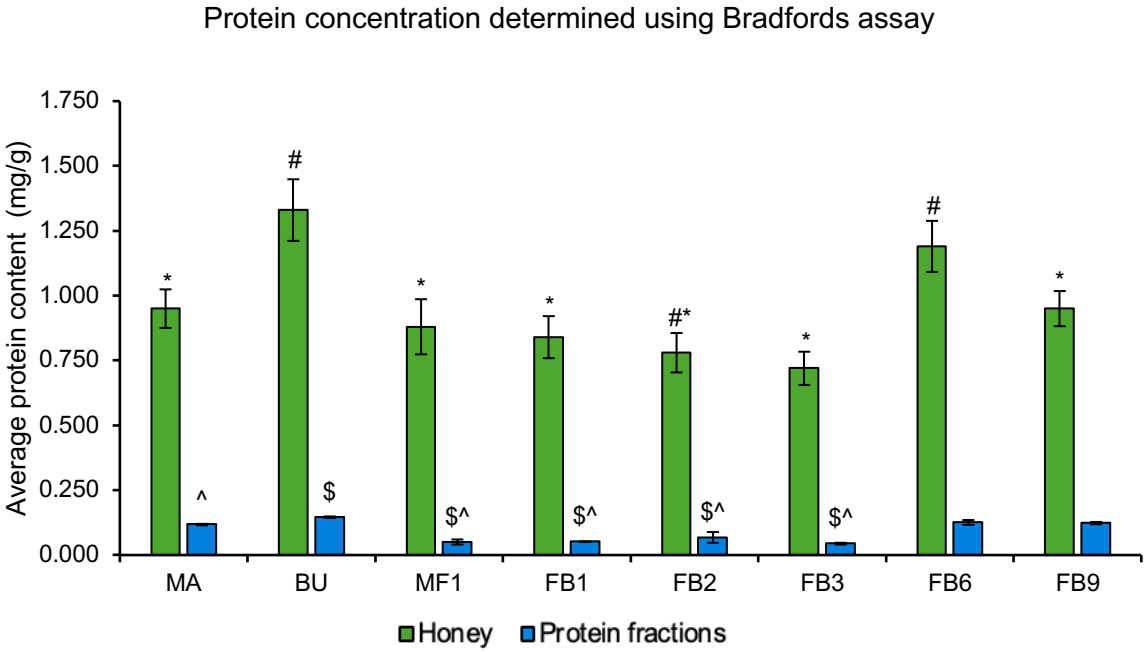


Figure 3.4.2. The average protein concentration (mg/g) of each 100% honey and the protein fraction. Data is an average of three experiments ± SEM. Data is normally distributed and statistical significance was determined using ANOVA tests. Samples denoted with # are significantly different to the MA whole honey control, * are significantly different to the BU whole honey control, $ are significantly different to the MA protein control and ^ are significantly different to BU protein control, p≤0.05.

For the honey samples the sugar/fructose content was 37.70 ± 0.17 mg/100 g for MA, 29.68 ± 0.055 mg/100 g for BU, 25.18 ± 0.10 mg/100 g for MF1, and the FB samples the range was from 29.21 ± 0.10 - 38.99 ± 0.18 mg/100 g (Table 3.4.3. and Figure 3.4.3). Differences compared with MA was significantly lower for MF1 (p=0.0212)

The sugar/fructose content of the protein fractions were 15.95 ± 3.15 mg/100 g for MA, 11.07 ± 0.63 mg/100 g for BU, 12.58 ± 1.48 mg/100 g for MF1, and for the FB samples ranged from 13.07 ± 0.70 - 22.01 ± 1.85 mg/100 g, respectively (Table 3.4.3 and Figure 3.4.3). Differences compared with BU was significantly higher for FB9 (p=0.0389).

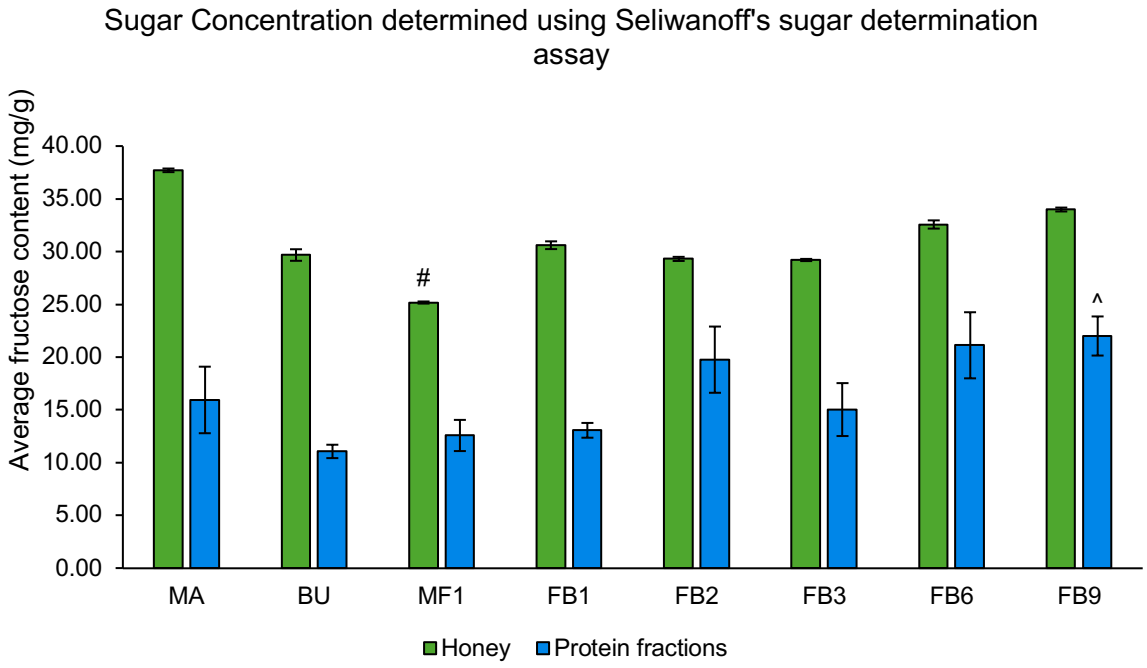


Figure 3.4.3. The average sugar/fructose sugar concentration (mg/g) of each 100% honey and protein fraction. Data is an average of three experiments ± SEM. Data is normally distributed and statistical significance was determined using ANOVA tests. Samples denoted with # are significantly different to the MA (control) and ^ were significantly different to the BU protein (control), p≤0.05.

The TPC for the whole honey was determined with the F-C assay. The TPC was 0.81 ± 0.21 mg GAE/g for MA, 1.05 ± 0.03 mg GAE/g for BU, 0.47 ± 0.04 mg GAE/g for MF1, and the range for the FB honey was 0.34 ± 0.02 - 0.92 ± 0.11 mg GAE/g (Table 3.4.3). Relative to MA, the TPC of BU (p=0.0273) was significantly increased, whereas for MF1 (p=0.0002), FB2(0.34 ± 0.02 mg/g, p=<0.0001) and FB3 (0.36 ± 0.02 mg/g, p=<0.0001) was reduced (Figure 3.4.4). The TPC of FB6 was 0.92 ± 0.11 mg GAE/g, which was higher than that of MA (p=0.8223) and lower than that of BU (p=0.5617). However, no statistically significant difference was detected, indicating similarity in their TPC values.

Gel filtration chromatography was used to separate the small molecules such as the polyphenols from the peptides and proteins (Figure 3.4.4).

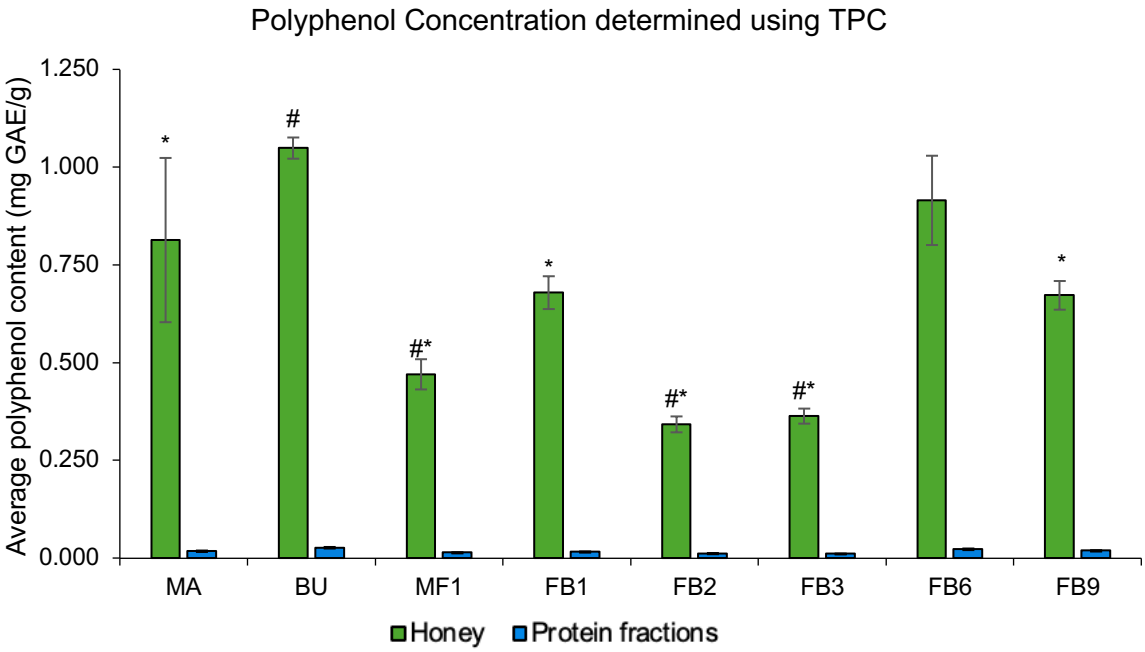


Figure 3.4.4. Total polyphenol content (mg GAE/g) of the 100% honey and the protein fractions. Data is an average of three experiments ± SEM. Data is normally distributed and statistical significance was determined using ANOVA tests. Samples denoted with # significant differences relative to MA (control) and * significant differences relative to BU (control), p≤0.05.

The physiochemical properties of the honeys and the protein fractions are summarised in Table 3.4.3. The darkest honey was BU, with the highest TPC. The honey with the highest fructose content was FB9. Of the South African honeys, including MF1, the darkest was FB6, which also had the highest TPC concentration.

The colour and the TPC for all honeys were correlated (Figure 3.4.5). The Pearson correlation was r = 0.7435 indicating the darker the honey the higher the TPC. If sample BU result is removed (due to potential assay limitations) the correlation increases to r = 0.8116 indicating that the colour of BU is not only due to the presence of polyphenols.

Table 3.4.3. Physiochemical properties of the honey samples and the relative amounts of fructose, and polyphenols in the protein fractions.

Parameter	Sample	MA	BU	MF1	FB1	FB2	FB3	FB6	FB9
Protein (mg/g)	Honey	0.95 ± 0.07	**1.33 ± 0.12**^{aaaa}	0.88 ± 0.11	0.84 ± 0.8^{a}	0.78 ± 0.08^{aaaa}	0.72 ± 0.06^{aaaa}	1.19 ± 0.1^{aaaa}	0.95 ± 0.07
	Protein	0.12 ± 0.01	**0.15 ± 0.01**^{b}	0.05 ± 0.01^{bbbb}	0.05 ± 0.01^{bbbb}	0.07 ± 0.02^{bbbb}	0.04 ± 0.01^{bbbb}	0.13 ± 0.10	0.12 ± 0.01
Fructose (mg/100 g)	Honey	37.70 ± 0.17	29.68± 0.55	25.18 ± 0.10^{a}	30.61 ± 0.37	29.31 ± 0.20	29.21 ± 0.10^{aa}	32.57 ± 0.39	**38.99 ± 0.18**
	Protein	15.98 ± 3.15	11.07 ± 0.63	12.58 ± 1.48	13.07 ± 0.70	19.76 ± 3.14	15.04 ± 2.51	21.13 ± 3.13	**22.01 ± 1.85**
TPC (mg GAE/g)	Honey	0.81 ± 0.21	**1.05 ± 0.03**^{aaa}	0.47 ± 0.04^{aaaa}	0.68 ± 0.04	0.34 ± 0.02^{aaaa}	0.36 ± 0.02^{aaaa}	**0.92 ± 0.11**	0.67 ± 0.04
	Protein	0.018 ± 0.01	**0.03 ± 0.01**	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
A_{450} (AU)	Honey	0.27 ± 0.02	**1.87 ± 0.02**^{aaaa}	0.04 ± 0.02	0.40 ± 0.02^{aaaa}	0.14 ± 0.01^{aaaa}	0.21 ± 0.01	**0.51 ± 0.02**^{aaaa}	0.29 ± 0.02
Colour* (AU)	Honey	0.20 ± 0.01	**1.59 ± 0.01**^{aaaa}	0.21 ± 0.01	0.33 ± 0.01^{aaaa}	0.10 ± 0.01^{aaaa}	0.17 ± 0.01	**0.44 ± 0.03**^{aaaa}	0.23 ± 0.01

*(A_{450}-A_{720}), Bold= samples with the highest concentrations
Statistical significance compared to the MA honey(a) or protein(b) control is indicated as
P<0.05^{a} or ^{b}
P<0.01^{aa} or ^{bb}
P<0.00^{aaa} or ^{bbb}
P<0.0001^{aaaa} or ^{bbbb}
A450 and colour of protein fractions was not included in data set

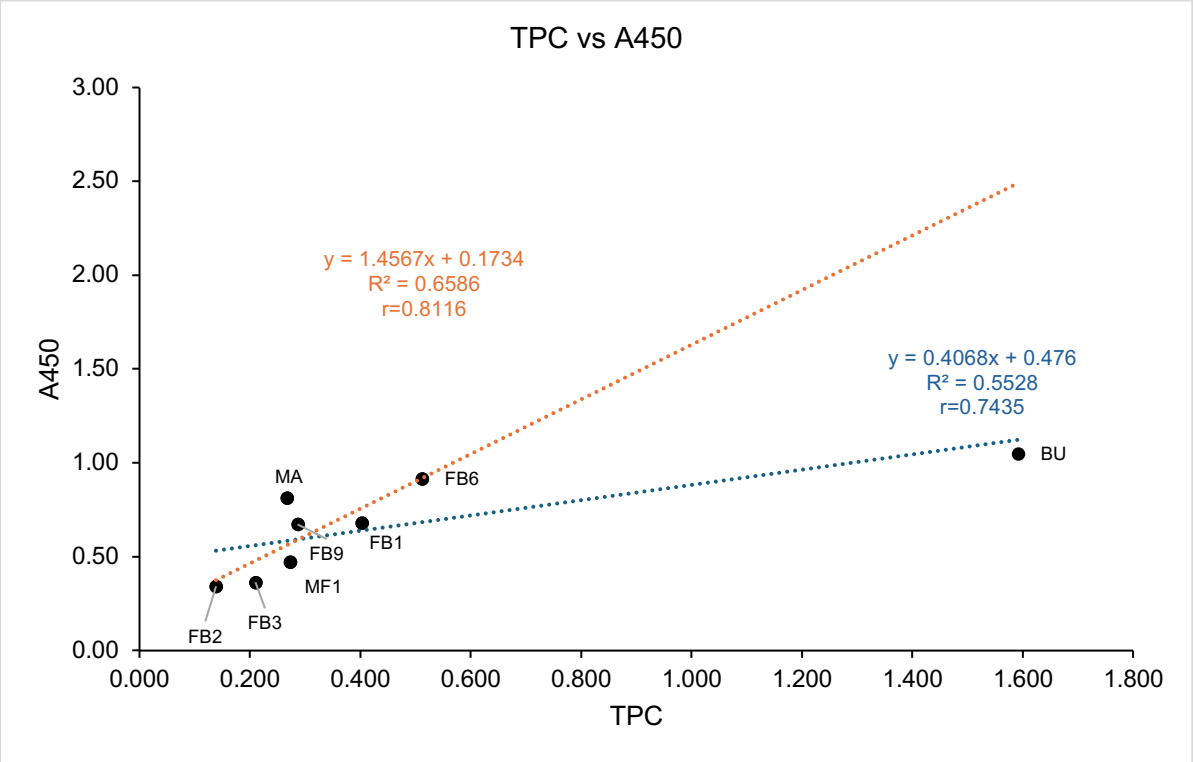


Figure 3.4.5. The correlation between A_{450} (AU) and TPC (mg GAE/g) of honey samples, the orange line represents the same correlation without BU, the equation of the line, y= 1.4567x + 0.1734, R^2=0.6586, and r= 0.8116. Significance for both lines was determined using ANOVA testing on Microsoft Excel, where p=0.0344 for the blue line and p=0.0266, determining significant* correlation for both lines.

3.4.4 Molecular mass profile determination

SDS-PAGE analysis confirmed the presence of proteins in the isolated fractions. The molecular mass profiles of the honey samples and protein fraction had distinct intensive protein bands ranging from 45-70 kDa, (Figure 3.4.6, Table, 3.4.4). In all the protein fractions, two prominent bands between 50 and 75 kDa with masses of ± 50.2 - 68.6 are observed. In BU honey there is a low molecular mass band of ± 9.4 kDa that is also present in FB2. BU contains a prominent band of approximately 24.7 kDa, which is shared and most distinct in samples FB3, FB6, and FB9.

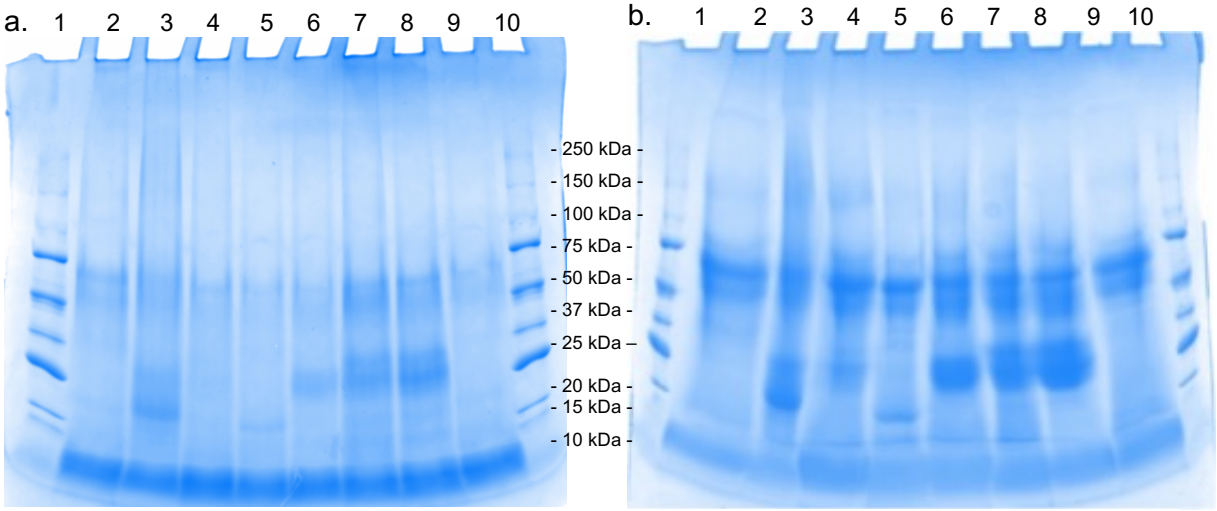


Figure 3.4.6. Representative images of 4-20% SDS-PAGE Tris glycine gels loaded with (a) 15 µg whole honey and (b) 2 µg protein. Lane 1 and 10 contain the molecular mass standards (Precision Plus Protein™ unstained standard), lane 2, MA, lane 3, BU, and lanes 4-8, FB1, FB2, FB3, FB6, and FB9 respectively, with lane 9 containing MF1.

The protein identification analysis of honey samples and their isolated protein fractions was conducted using SDS-PAGE, with probable protein identifications assigned based on literature references. The first table (Table 3.4.4) presents the molecular weight distribution of protein bands detected in whole honey samples, with the most intense bands appearing at approximately 50–75 kDa across multiple samples. Notably, BU exhibited a diverse range of proteins, including defensin-1 (DF-1), apolipophorin-III-like proteins, and major royal jelly proteins (MRJP), highlighting its rich protein composition.

The second table (Table 3.4.5) details the protein fractions isolated from honey, demonstrating a more refined identification of proteins. Several samples contained histone H4, chymotrypsin inhibitors, and venom serine proteases, suggesting potential bioactive properties. The presence of GOx across multiple samples aligns with honey's antimicrobial activity. Interestingly, FB6 and FB9 shared similar protein profiles, indicating possible compositional overlap. The identification of MRJPs and DF-1 in both whole honey and isolated fractions reinforces their significance in honey's functional properties.

Table 3.4.4. Protein bands detected in the honey samples analysed using SDS-PAGE, the protein bands and retention factor (Rf) were identified using ImageLab (BioRad, Lasec, Western Cape, SA), *probable identification of the protein or peptide types were done according to identification conducted by Erban *et.al*.[7]

	Molecular weight (kDa)										
Honey	**<10**	**10-15**	**15-20**	**20-25**	**25-37**	**37-50**	**50-75**	**75-100**	**100-150**	**150-250**	***Probable identification**
MA											DF-1, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
BU											DF-1, icarapin (Api m10 allergen), apolipophorin-III-like, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
MF1											DF-1, venom serine protease, MRJP 9, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
FB1											DF-1, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
FB2											DF-1, hymenoptaecin, histone H4, ubiquitin 60S ribosomal protein L40, chymotrypsin inhibitor, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
FB3											DF-1, icarapin (Api m10 allergen), apolipophorin-III-like, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
FB6											DF-1, icarapin (Api m10 allergen), apolipophorin-III-like, venom serine protease, MRJP 9, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
FB9											Same as FB6

Dark orange indicates the most intense and light orange the least intense bands.

Table 3.4.5. Protein bands detected in the protein fractions using SDS-PAGE, the protein bands and retention factor (Rf) were identified using ImageLab (BioRad, Lasec, Western Cape, SA), *probable identification of the protein or peptide types were done according to identification conducted by Erban *et.al.*[7]

	Molecular weight (kDa)										
Protein	**<10**	**10-15**	**15-20**	**20-25**	**25-37**	**37-50**	**50-75**	**75-100**	**100-150**	**150-250**	***Probable identification**
MA											DF-1, hymenoptaecin, histone h4, ubiquitin 60S ribosomal protein L40, chymotrypsin inhibitor, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
BU											DF-1, uncharacterised proteins LOC408608 and LOC726323, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
MF1											DF-1, hymenoptaecin, histone h4, ubiquitin 60S ribosomal protein L40, chymotrypsin inhibitor, venom serine protease, MRJP 9, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
FB1											DF-1, uncharacterised proteins LOC408608 and LOC726323, icarapin (Api m10 allergen), apolipophorin-III-like, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase xanthine dehydrogenase, reductase (short chain), lysosomal- α -mannosidase
FB2											DF-1, hymenoptaecin, histone h4, ubiquitin 60S ribosomal protein L40, chymotrypsin inhibitor, venom serine protease, MRJP 9, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase, xanthine dehydrogenase, reductase (short chain), lysosomal- α -mannosidase
FB3											DF-1, uncharacterised proteins LOC408608 and LOC726323, venom serine protease, MRJP 9, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
FB6											DF-1, icarapin (Api m10 allergen), apolipophorin-III-like, venom serine protease, MRJP 9, MRJP2-5, MRJP7, GOx, α-glucosidase III, α-amylase
FB9											Same as FB6

Dark orange indicates the most intense and light orange the least intense bands.

3.5 Discussion

3.5.1 Honey adulteration in a South African context

Honey's chemical fingerprint is intricately tied to its production environment, encompassing factors such as geographical location, botanical sources, and manufacturing processes. In South Africa approximately half of the honey originates from *Eucalyptus* spp, citrus, Fynbos (*Erica* spp) and sunflower sources.[189] The Fynbos biome, situated in the Western Cape (WC), stands out as one of the most diverse flora regions with around ±7000-9000 endemic flora species.[190] South Africa is a modest honey-producing nation, with only 10% of beekeepers operating at a commercial scale (8000-10000 hives). The majority (90%) of honey is produced by hobbyist beekeepers with fewer than 100 hives. South Africa's annual yield is approximately 1500 tons, satisfying only 50% of the market demand for honey. To meet the remaining 50%, honey is imported from China, Argentina, and Romania.[189] Research conducted by Zhou *et. al.*[191] analysed 100 honey samples from 19 different botanical sources, revealing adulteration particularly prevalent in commercial honey. Notably, 52% of adulterated samples originated from Asia, 28% from Europe, and 18.4% from Australia.[189,192] Consequently, understanding the physiochemical properties of honey is essential for assessing its quality, composition and safeguarding against potential adulteration.[189]

3.5.2 Physiochemical determination

The fructose content is an important sugar to test for as it is the predominant sugar found within honey accounting for majority of the sweet taste associated with the nutraceutical.[193] The fructose content within the honey samples was 25.18 ± 0.10 - 38.99 ± 0.18 mg/100 g identified in this study, lower than that identified by Nguyen *et. al.*[194] who had a fructose concentration of 44.2 ± 2.0 mg/100 g.[194] Furthermore, the Codex Alimentarius and South African Department of Agriculture determines the combined fructose content of honey to be between 27200-44300 mg/100 g (272-443 mg/g) which is higher than that of our honey and protein samples used in this study.[195] This suggests that the honey quality or authenticity is compromised, however, such a discrepancy suggests the possible degradation of fructose, influenced by environmental factors, including the presence of metals, or through thermal degradation during the sterilisation process of the honey samples. These findings highlight the importance of proper honey processing and storage in preserving the sugar content.[196,197]

Regarding the analytical methods, the Seliwanoff's assay, used in this study, specifically detects ketoses (e.g., fructose) and distinguishes them from aldoses.[184] It relies on a reagent (resorcinol and H_2SO_4) that reacts preferentially with ketoses, leading to a distinct colour

change (cherry red).[184] In contrast, the Benedict's assay, a more commonly used method that detects a broader range of reducing sugars (e.g., glucose), relying on the reduction of Cu^{2+} ions to Cu^{1+}, producing a colour spectrum from green to red depending on sugar concentration.[177] Since the Seliwanoff's assay does not account for protein interference (which also relies on the reduction of Cu^{2+} ions to Cu^{1+})[178] unlike Benedict's assay,[177] variations in measured sugar concentrations may result from differences in detection methods. Furthermore, Benedicts test is widely preferred in research as it is considered better to use than Seliwanoffs test due to its ability to detect a wider range of reducing sugars in a complex sugar model such as honey, its simplicity, and accessibility to reagents.[178] However, there is a lack of supporting evidence to show the limitations and benefits of either test especially on honey sources of different geographical origins, with varying protein compositions, and how these proteins interfere with either test. Therefore, for the purposes of this study making use of an HPLC method to detect sugar composition such as that described by Aljohar *et. al*.[198] would provide a more accurate profiling of the sugar content in our honey samples. Additionally, this study was limited by the lack of analysis of other key sugars in honey, such as glucose and sucrose. Future studies should include these sugars and consider using an artificial honey solution with a known concentration of fructose, glucose, and sucrose as an analytical control to enhance accuracy and reliability.

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The TPC of all honey samples ranges from 0.34 ± 0.02 -1.05 ± 0.03 mg GAE/g. Honey samples MA, BU, and FB6 had TPC values of 0.81 ± 0.21 mg GAE/g, 1.05 ± 0.03 mg GAE/g, and 0.92 ± 0.11 mg GAE/g (Table 3.4.1), respectively. These samples showed higher TPC values compared with 0.72 mg/g for a Manuka honey sample identified by Nguyen *et. al*.[194] Similarly, samples MA, BU, FB1, FB6, and FB9 had TPC values (Table 3.4.1) higher than the 0.56 mg/g TPC identified for a Manuka (AAH 8+) honey sample identified by Deng *et. al*. [103] A study conducted by Esterhuizen *et. al*.[195] identified the TPC content of South African West coast (Fynbos biome) honeys ranging between 0.00-1.03 mg/g, the Fynbos honeys used in this study had a TPC range of 0.34 ± 0.02 - 0.92 ± 0.11 mg GAE/g which falls within the same range reported by Esterhuizen *et. al*.[195] Moreover, Fynbos honey samples FB1, FB6, and FB9 showed no statistically significant difference to the MA control, indicating a similarity in TPC compared with the Manuka control. Magoshi *et. al*[199] identified the TPC of FB honeys at an average of 0.56 ± 0.07 mg GAE/g which is similar to the average of the FB honeys at 0.59 mg GAE/g reported in this study. The Codex Alimentarius and South African Department of Agriculture recommends a TPC of honey samples ranging between 0.05 -13.00 mg/g, the TPC's of all honey samples used in this study fall within this regulatory range.[195]

The colour of the honey samples ranged from 0.10 ± 0.01 - 1.59 ± 0.01 AU (Table 3.4.3). For MA honey, the absorbance was 0.20 ± 0.01 AU slightly lower than reported for a Manuka sample with 0.220 AU.[194] Sample BU, FB1, and FB6 at 1.59 ± 0.01 AU, 0.33 ± 0.01 AU, and 0.44 ± 0.03 AU, significantly higher than the MA control at 0.20 ± 0.01 AU (Table 3.4.3). Samples FB9 and MF1 at 0.23 ± 0.01 AU and 0.21 ± 0.01 AU, respectively, were similar to the MA control (Table 3.4.3). The correlation for TPC and A450 was 0.7435 (Figure 3.4.6) indicating a strong correlation indicating that the darker the honey the higher the polyphenol content. For example, BU which had the highest A_{450}, colour, and TPC at 1.87 ± 0.02 AU, 1.59 ± 0.01 AU, and 1.05 ± 0.03 mg GAE/g (Table 3.4.3), respectively. Interestingly, when BU was excluded from the correlation analysis the correlation increased to 0.8116, indicating a greater similarity between MA and the FB honeys.

3.5.3. Protein determination and tentative identification

The protein content of all honey samples ranges from 0.72 ± 0.06 - 1.33 ± 0.12 mg/g and of all the protein fractions was 0.04 ± 0.01 - 0.15 ± 0.01 mg/g (Figure 3.4.2, Table 3.4.1). Honey samples FB6 and BU had comparable protein concentrations compared to that reported for a commercial Manuka honey (MGO=400+ mg/kg) at 1.29 mg/g determined by Nguyen *et. al.*[194] Furthermore a study conducted by Deng *et. al.*[103] identified the protein content of a Manuka honey (AAH 8+) sample, from Canterbury, New Zealand, to be 0.63 ± 0.01 mg/g, slightly lower than that identified for all honey samples used in this study.[103] The studies done by Nguyen *et. al.*[194] and Deng *et. al.*[103] used the same Bradford's method[181] described in this study however they incubated for 10 minutes prior to reading the absorption.[194] The binding of the dye to protein is a very rapid process (approximately 2 min), and the protein-dye complex remains dispersed in solution for a relatively long time (approximately 1 h), making the procedure rapid and yet not requiring critical timing for the assay to successfully yield results.[181] Therefore quantitative differences in the protein concentrations between the studies conducted by Nguyen *et. al.*,[194] Deng *et. al.,*[103] and this study are not due to differences in methods.

Unfortunately, the Codex Alimentarius and South African Department of Agriculture do not define protein concentration range, therefore there is no official quality standard for the protein concentration in honey, reiterating the need for honey protein research.[200] The similarity in protein concentration between samples FB6 and BU and the commercial Manuka honey (MGO = 400+ mg/kg) analysed by Nguyen *et. al.*,[194] along with the higher protein content compared to the Manuka honey (AAH 8+) sample from Canterbury, New Zealand, studied by

Deng *et. al*.,[103] suggests a similarity in the foraging regions of the respective bee species. Since honey proteins originate primarily from pollen sources specific to plant species and the hypopharyngeal glands unique to each bee species,[201] these findings may indicate comparable protein profiles between medically significant Manuka honey and South African Fynbos honey samples. This similarity could serve as a basis for similar anti-inflammatory, antioxidant, and antimicrobial activity, potentially driven by the protein components present in both honey types. Therefore, to further confirm similarity in the protein profiles of the Manuka honey sample used in this study and the Fynbos samples SDS-PAGE was used.

The SDS-PAGE analysis indicated clearly detectable protein bands for the honey samples ranging from 6.27-132.36 kDa. The most intensive protein bands for all honey samples ranged between 50 - 70 kDa (Figure 3.4.6a). There was a unique protein band found in honey samples and protein fraction of BU and FB2 at ± 9.43 kDa (Table 3.4.4). A study conducted by Lewkowski *et. al.*[156] identified the protein bands within honey using mass spectrometry of proteins cut out from SDS-PAGE gels and was cross referenced to the known *A. mellifera* proteins. Bands ranging from 50-70 kDa were predominantly MRJPs.[156] The theoretical molecular mass profiles for MRJP1 ranges from 48-55 kDa, MRJP 2 ranges from 49-51 kDa, MRJP3 ranges from 59-62 kDa, MRJP5 ranges from 68-70 kDa, and MRJP7 ranges from 48-51 kDa.[202,203] The MRJPs have been linked to anti-inflammatory activity, antimicrobial activity, antioxidant activity, wound healing, and cell proliferation.[203]

Little is known regarding the proteins present in the honey and this includes the type and concentrations as well as the contribution of the fraction to the bioactivity of honey. In this study gel filtration chromatography was used to isolate the protein fraction. [7] For all honey samples, the proteins were separated from the honey samples (Table 3.4.2). The protein concentration was 0.04 ± 0.01 – 0.15 ± 0.01 mg/g. Analysis of the protein content indicates that the recovery of the proteins from the honey samples was low (Figure 3.4.2). This is in contrast to the 0.1-1.4 mg/g reported by Erban *et. al.*[7] who isolated the protein fractions using a similar gel filtration chromatography technique that employed the same Sephadex G-25 stationary phase and determined their protein concentrations using the same Bradfords method.[181] Although the gel filtration chromatography technique was similar, it was coupled with a state-of-the-art Orbitrap Fusion Tribrid mass spectrometer used to identify the isolated fractions with protein hits assigned taxonomically to the honey bee species.[7] Mass spectrometry works by ionising molecules in a sample, separating those ions based on their mass to charge ratio using a mass analyser such as the Orbitrap.[204] Subsequently, these molecules are quantified and identified based on their unique mass, making it a highly

sensitive technique for protein identification by analysing the masses of peptides generated from protein digestion.[204] Therefore the difference in protein concentration of the isolates was due to the differences in the sensitivity of selection techniques and subsequent pooling of fractions with the highest protein content. Nonetheless, the fructose, and polyphenol of the protein fractions was very low indicating the selection of a fraction free of these molecules as contaminants. With SDS-PAGE the proteins in the protein fractions were tentatively assigned based on molecular mass and intensities.

The SDS-PAGE analysis indicated clearly detectable protein bands for the protein fraction ranging from 6 - 70 kDa. The most intensive protein bands for all protein fraction ranged between 50- 70 kDa (Figure 3.4.6b). There was a unique unassigned protein band found in the BU and FB2 honey samples and protein fractions at ± 9.43 kDa (Table 3.4.4 and 3.4.5). Studies conducted by Paget *et. al.*[205] and Bong *et. al.*[206] used mass spectrometry analysis to confirm the presence of the MRJP family in 12 Manuka and 42 Manuka honeys, respectively.[205,206] All protein fractions had clearly detectable intense protein bands between 50 - 70 kDa which falls within the theoretical range of the MRJPs in honey, indicating the presence of MRJP protein family in the honey samples. Furthermore, GOx has a theoretical molecular mass of ± 67 kDa which is also within the range of intensive staining protein bands between 50 - 70 kDa for both the honey samples and protein fractions in this study.[202] DF-1 has a theoretical molecular mass of 4-6 kDa, FB1, FB3, FB6, and FB9 have distinct protein bands at ± 6 kDa tentatively identified as DF-1.[140]

The MRJP protein family, GOx, and DF-1 are proteins of interest in the protein profiles of these samples due to their contributions to the antimicrobial activity of honey. MRJPs exert their antimicrobial properties by binding to bacterial cell walls, where their glycoproteins induce non-specific membrane permeabilization causing structural damage and lysis, particularly in Gram-positive bacteria.[143] Additionally, MRJPs inhibit aerobic respiration and cell division contributing to bacterial cell death.[207] Complementing these effects, GOx catalyses the oxidation of glucose to produce H_2O_2, which has potent antimicrobial properties by damaging the bacterial DNA and proteins.[207] Another key mechanism originates from DF-1, which is a positively charged antimicrobial peptide that interacts with the negatively charged phospholipids of the bacterial cell membrane.[144,208] This interaction creates small pores in the membrane that disrupt bacterial cell integrity, causing leakage of key cellular components and ultimately bacterial cell death.[144,208]

Although SDS-PAGE is a widely used and understood technique in protein analysis, it is important to recognise the limitations of this techniques. These limitations include, the occurrence of glycosidic protein products, protein crosslinking, proteolytic degradation during sample preparation with a resultant loss of protein bands, poor mass resolution multiple proteins within a single well defined band and a sensitivity bias for the more abundant proteins.[209] Additionally, the bands on the SDS-PAGE gels are representative of multiple proteins at an estimated molecular weight, therefore it is recommended for future studies that further honey fingerprinting is conducted using LC-MS/MS analysis[210] against known proteins for *A. mellifera* to accurately determine and confirm the presence of the MRJP protein family, GOx and/or bee DF-1.

3.6 Conclusion

In this study, compared with MA, the TPC of the FB honeys were similar with a high correlation between the colour and TPC. The honey samples and protein fractions had similar predominant proteins following isolation using gel filtration chromatography, these bands can be aligned with the MRJP protein family, GOx, and bee-defensin-1. However, further honey fingerprinting is needed using LC-MS/MS analysis[210] against known proteins for *A. mellifera* to accurately determine and confirm the presence of the MRJP protein family, GOx and/or DF-1. In the protein fractions, the sugar and polyphenol content was low, indicating effective isolation of the protein fraction. Subsequent SDS-PAGE identified the presence of those proteins, the MRJP protein family, GOx, and DF-1 identified in the honey samples.

Chapter 4: The cytotoxicity and cell migratory activity of selected South Africa Fynbos honeys and protein extracts

4.1 Introduction

Cytotoxicity analysis is critical for *in vitro* research providing insights into the effect a drug/nutraceutical elicits on cell viability and function. Not only is this type of analysis an aid during drug discovery research, it also acts as a reliable alternative to *in vivo* testing during preliminary assessments ultimately minimising ethical concerns and resource constraints.[211] Evaluation of cytotoxicity can identify the potential toxic concentrations or nature of a substance before moving forward to complex and costly animal models. [212] *In vitro* methods are on the whole more cost effective allowing for minimal sample and reagent use, subsequently producing high throughput screening suitable for assessing several drugs/nutraceuticals simultaneously.[212] Furthermore, understanding the mechanism of action of the cytotoxic assay used, for example membrane damage or disruption of protein/DNA synthesis, is crucial for determining a safety and efficacy profile of the compound/s being tested.[213]

Two cell lines were used in this study, namely, the human keratinocyte (HaCaT) and the murine fibroblast (SC-1) cell line. Keratinocytes and fibroblasts are key cellular role players in the post-inflammatory phase of cutaneous repair and regeneration, responding to inflammatory signals that activate proliferation and maturation of these cells essential for the wound healing process.[214] Keratinocytes are the dominant cell type found in the epidermis, acting as executers of the re-epithelialisation process by migrating, proliferating and differentiating to restore the epidermal barrier.[215] The fibroblasts are responsible for aiding in normal wound healing through their role in fibrinolysis, synthesising new ECM components and collagen, and wound contracture.[216] Interestingly, keratinocytes and fibroblasts synergistically communicate through double paracrine signalling loops coordinating the actions of these cell types to restore homeostasis.[214] Fibroblasts secrete paracrine signals such as basic fibroblast growth factor (bFGF), bFGF-2, VEGF-A, insulin-like growth factor-1 (IGF-1), and keratinocyte growth factor (KGF/bFGF-7) signalling to nearby keratinocytes. Additionally, responding to paracrine signals from keratinocytes and inflammatory cells, fibroblasts synthesise collagen and promote cross-linking to form an extracellular matrix, and differentiate into myofibroblasts facilitating wound contracture.[214,216] Due to the crucial roles keratinocytes and fibroblast play in cutaneous wound healing, they were selected to be used for experimentation in this study.

Several different assays can be used to quantify the effects on cell viability and the SRB cytotoxicity assay is the preferred method as it is recommended by the National Cancer Institute (USA) due to its increased linearity, higher sensitivity, cost effectiveness, and stable end point measurement.[217] The SRB dye binds to the basic amino acids in cellular proteins and DNA under mild acidic conditions providing a colorimetric measurement of the protein mass that directly correlates to the cell number.[217]

In contrast, cellular proliferation, required for wound healing can be evaluated with the scratch assay using adherent cell lines such as fibroblasts, epithelial and endothelial cells.[218,219] Several methods to induce a physical scratch or disruption of the 2D culture include toothpicks, pipette tips, cell scrapers, electric currents, and lasers.[218] A common characteristic of this method is the use of bright-field microscopy imaging to analyse the process of cell migration into the cleared zone at pre-determined time points and analysis of the rate and extent of gap closure in an image software.[218]

Essential requirements for effective wound healing mediated by medicinal honeys are the lack of toxicity and the potential for increased cell migration.

Chaudhary *et. al.*,[220] Sell *et. al.*[221] reported a time dependent *in vitro* migratory effects of 1% (v/v) Manuka honey on human fibroblast cells (HDF), in a study by Bucekova *et. al.*[139] a dose dependent relationship between DF-1 and MMP-9 secretion promoted the migration of keratinocytes *in vitro*.[139] Ranzato *et. al.*[222] identified the ability of Manuka, Buckwheat, and Acacia honeys to up-regulate or down-regulate regulatory genes in epithelial-mesenchymal transition and re-epithelialisation thereby promoting keratinocyte migration.[222] These studies indicate that Manuka, Buckwheat and Acacia honey promote cell migration. In the present study, in addition to determining the cytotoxicity, the potential of FB honey to promote cell migration was determined.

The aim of the research undertaken in this chapter was to evaluate the cytotoxicity and the cell migratory effects of the selected South Africa Fynbos honeys and protein fractions.

The specific objective for this chapter was:

1. To determine the cytotoxicity and cell migratory effects of both the whole honey and protein fractions on human keratinocytes (HaCaT) and murine fibroblasts (SC-1) using the SRB assay and the scratch migration assay.

4.2 Materials

Honey samples

The same honey samples stated in Chapter 3, were used in these assays.

Reagents, equipment and disposable plasticware

Reagents used are the same as in Chapter 3 and in addition: Dulbecco's Modified Eagle Medium (DMEM) (4.5 g/L glucose, L-glutamine, sodium pyruvate, and 3.7 g/L $NaHCO_3$), foetal calf serum (FCS) (Gibco, Thermo Fisher Scientific, Johannesburg, SA), penicillin/streptomycin/amphotericin B mix (10 000 U/mL penicillin, 10 mg/mL streptomycin, 20 µg/mL amphotericin B in 0.85% saline) (Pan Biotech, Biocom Africa (Pty) LTD, Gauteng, SA), TrypLE™ Express (1X) (Gibco, Thermo Fisher Scientific, Johannesburg, SA), Triton™ X-100, 100% acetic acid (glacial) (Merck, Modderfontein, SA), Sulforhodamine B (SRB) (Sigma Aldrich, Atlasville, SA), trichloroacetic acid (TCA)(Associated Chemical Enterprises (ACE), Johannesburg, SA), and Tris buffer pH 10.5 (Research Organics Inc, Cleveland, USA), trypan blue (Sigma Aldrich, Atlasville, SA).

Equipment used included those in Chapter 3 and in addition: 132A 40L water bath (Labotech), Gauteng, SA) Hermle Z300 centrifuge (Lasec, Gauteng, SA), Nuaire incubator (Lasec, Gauteng, SA), Synergy II (Biotek Instruments, Inc., Winooski, USA), Esco Laminar Flow Cabinet (Labotech, Gauteng SA), Olympus IX71 Microscope (Olympus Microscopes, Tokyo, Japan), AxioCam ERc5s camera, AxioCam vision software (Zeiss, Jena, Germany), and Fiji/ImageJ software (softonics), wound healing plugin tool (developed by Suarez-Arnedo *et. al.),*[223] and vertical type steam sterilizer (Labotech, Gauteng SA).

Disposable plasticware included: The same disposable plasticware listed in Chapter 3, was also used in the resrach undertaken in this chapter.

Cell lines

Immortalised human HaCaT keratinocytes (passage number 44-49) obtained from Cellonex, Johannesburg, SA. Murine SC-1 fibroblasts (passage number 5-9) were obtained from Highveld Biotech, Johannesburg, SA.

4.3 Methods

Cell culture

HaCaT (passage number 44-49) and SC-1 (passage number 5-9) cell lines were maintained in T_{75} flasks using supplemented DMEM (supplemented with 10% FCS and 1%

penicillin/streptomycin, at 37°C, 5% CO_2. Aseptic technique was maintained while working with the cell cultures. Supplemented DMEM was removed from the cell cultures, the cells were rinsed with 0.1 M sterile PBS. Cells were treated with 2-5 mL of TrypLE™ Express (1X) and incubated for ± 10 minutes until they were detached from the surface of the flask. A 2-5 mL volume of supplemented DMEM was added to the detached cell and TrypLE™ Express (1X) solution to deactivate the trypsin enzyme. The detached cells were collected using a pipette, transferred to a centrifuge tube, and centrifuged at 2000 g's for 2 minutes. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of fresh supplemented DMEM. The cells were counted using a haemocytometer and plated or split into separate T75 flasks, before incubation at 37°C with 5% CO_2.[224]

Sulforhodamine assay

The 48 hour cytotoxicity of the honey samples and protein fractions were investigated using HaCaT (human epidermal keratinocyte) and SC-1 (murine fibroblast) cell lines at a final concentration of 1.25% (v/v) and 0.625% (v/v) for the HaCaT and SC-1 cells respectively. Honey samples and protein fractions were prepared by filtering each sample using 0.45 uM Millipore syringe filters. The honey samples were further diluted in 0.01M PBS from a 25% (v/v) stock to 12.5% (v/v) and 6.25% concentration (1.25% (v/v) and 0.625 % (v/v) in well concentration). No dilution was required for the protein fractions. The SRB assay is a colorimetric method of quantifying cell viability and cell mass through the use of a pink/red fluorescent dye (SRB). The dye used to quantify the effects of exposure binds to basic amino acids, under acidic conditions, such as lysine, arginine, and histidine present in proteins and to DNA.[217] The amount of SRB dye that binds to the cells is directly proportional to the total protein content of the cells. After allowing sufficient time for dye binding, the excess SRB is washed away and the protein-bound SRB is extracted into a mild basic buffer and the absorbance is measures, the absorbance is directly proportional to the cell number.[225]

The SRB procedure followed has been adapted from Vichai *et. al.*[226] Cells treated with a final concentration of 0.01% Triton X 100 were the positive control, with 0.01 M PBS as the vehicle control/blank.

The HaCaT and SC-1 (doubling time of 28 h and 30 h, respectively)[227,228] cells were independently seeded at a concentration of 10 x 10^4 cells per well on a 96 well plate. 10 x 10^4 cells per well was the chosen seeding density as that is where the optimum confluency (~80%) in well was met after 24 h for both cell lines. The cells were allowed 24 h at 37°C and 5% CO_2 to adhere. After 24 h the HaCaT cells were exposed to 10 µL of the 12.5% (final concentration

1.25%) honey samples and 0.04-0.13 mg/g protein fractions (final concentration 0.0044-0.0126 mg/g) for 48 hrs. Both cell lines were treated for 48 h to observe a comprehensive effect of treatment after cell division, allowing for complete analysis of the treatment impact on cell growth and division.[229] Likewise the SC-1 cells were exposed, to both the 12.5% (final concentration 1.25%) and 6.25% v/v (final concentration 0.625% v/v) honey and 0.04-0.15 mg/g protein (final concentration 0.0044 - 0.0146 mg/g) for 48 h.

After incubation 50 µL fixative, 50% TCA solution was added to each well and the plates were placed fridge at 4°C for ± 16 h. Then the plates were washed under gentle flowing water until all residual TCA was removed and plate placed in the oven at 30°C until dry. To the individual wells of the dried plates, 100 µL of 0.057% SRB dye (57 mg of SRB stain in 100 mL 1% acetic acid) was added to each well and incubated at room temperature for 30 mins in the dark. The plates were then washed with 150 µL of 1% acetic acid until all residual SRB dye was removed and the plates again dried. To the dry plates, 200 µL of 10 mM Tris buffer solution (pH 10.5) was added to each well. The plates were left on a shaker for 1 h to extract the attached SRB dye. The absorbance was measured using the Biotek Synergy II spectrophotometer at 540 nm with a reference wavelength of 630 nm. The percentage cell viability was calculated using the following equation:

$$\% \text{ viability} = [abs_{sample} - abs_{blank} / abs_{control} - abs_{blank}] \times 100$$

Scratch assay

The migratory effects of the honey samples and protein fraction was investigated using HaCaT and SC-1 cell monolayers to determine if at the identified non-cytotoxic concentrations cell migration is promoted. This was undertaken using the scratch assay adapted from Martinotti *et. al*.[230]

Cells were treated with 150 µL of 10% FCS (final concentration 2.5%) as a positive control, and 0.01 M PBS as a vehicle/negative control. The HaCaT and SC-1 cells were seeded at 20 x 10^4 cells per well in 24 well plates. 20 x 10^4 cells per well was the chosen seeding density as that is where the optimum confluency (~80%) in well was met after 24 h for both cell lines. The cells were allowed to adhere for 24 h at 37°C and 5% CO_2. After 24 h the HaCaT and SC-1 cell monolayers were scratched using the long axial tip of a 1 mL pipette tip perpendicular to the bottom of the well. The scratch was made in a straight line following one direction, after which they were observed under the Olympus IX71 Microscope to ensure consistency of scratch size and shape across all wells. The scratched cell monolayers were imaged without

any staining using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software, all images were taken at T0.

The HaCaT and SC-1 scratched cell monolayers were exposed, in duplicate, to 50 µL of 0.04-0.15 mg/g (final concentration 0.0044 - 0.0146 mg/g) protein, 12.5% (v/v) (final concentration 1.25% (v/v)) honey for HaCaT cells, and 6.25% (v/v) (final concentration 0.625% (v/v)) honey for SC-1 cells for 24 h in 450 µL of serum free DMEM. After 24 h the HaCaT or SC-1 cells were fixed and stained with the Crystal Violet (CV) staining procedure. A 24 h exposure time was used to as lifting was observed in the cellular monolayer after 48 h exposure.

CV staining

To each well 50 µL of 20% formaldehyde (final concentration 5%) was added to each well and left to incubate at 37°C for 30 minutes. Once incubation was complete the supernatant was discarded, the plate was washed using tap water and left for dry. Once dry 100 µL of 0.1% (w/v) CV dye (prepared in 0.75% (v/v) formic acid at pH 3.5) was added to each well of the 24 well plate and left at room temperature for 30 minutes. The dye solution was then discarded, the plate was washed using tap water and left to dry.

The dried plates were then imaged using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software, all images were taken at T24, (4x magnification) following CV staining. The images were then analysed using the Fiji/ImageJ software to quantify cell migration after 24 h. The wound healing plugin tool developed by Suarez-Arnedo *et. al.*[223] (image: 8-bit, variance: 20, threshold: 50) was used to determine the area of the scratch in pixels at T0 and T24 to determine the wound closure rate using the following calculation described by Suarez-Arnedo *et. al.*[223] and Suh *et. al.*[231]

$$\textbf{\% wound closure} = (A_{T0} - A_{T24} / A_{To}) \times 100$$

Where A_{T0} is the wound area measured right after the scratch was performed, and A_{T24} is the area of the wound measured at the 24 h predetermined time.

<u>Data management and statistical analysis</u>

Data is an average of three experiments, for the cytotoxicity analysis, where each measurement was done in triplicate, subsequently, 9 data points were generated per sample (independent variable) treated as dependent variables. For the migration analysis, data is an average of duplicate experiments, due to resource constraints, where each measurement was

done in triplicate, subsequently, 6 data points were generated per sample (independent variable) treated as dependent variables. All data was expressed as mean ± SEM of the triplicate experiments analysed using Microsoft 365 Excel 2023. Determination of parametric and non-parametric data was conducted using the D'Agostino and Pearson test; and Shapiro-Wilk test using the GraphPad prism software version 9.5.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Data was statistically analysed using one way ANOVA and Tukey's multiple comparisons test was conducted for comparison of means using the same statistical software.

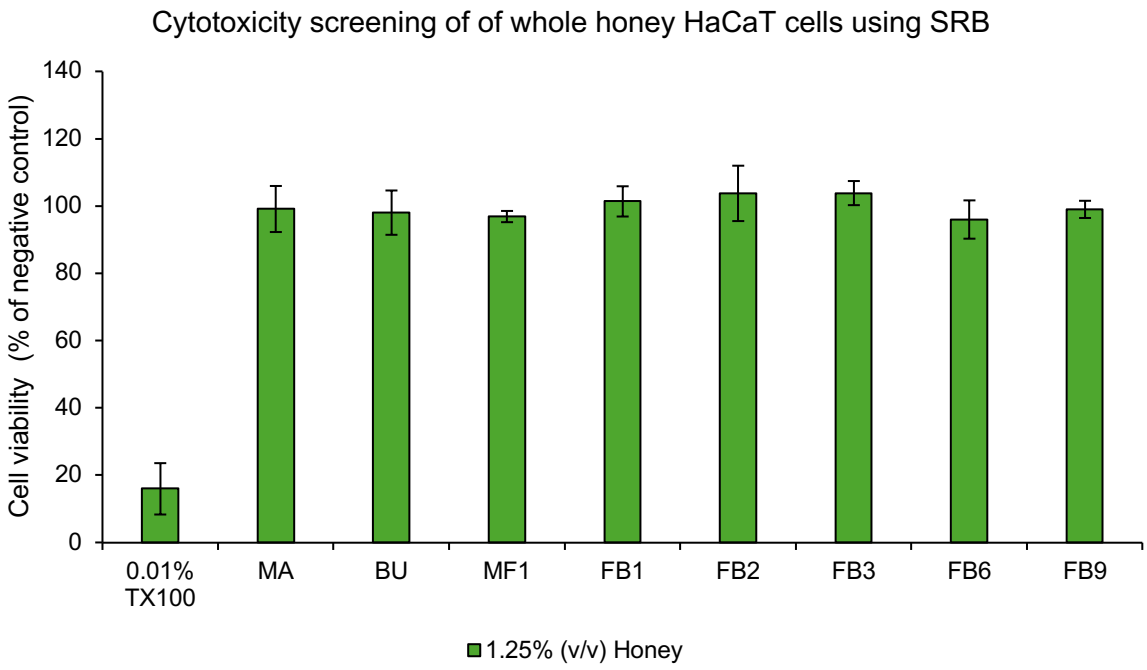
4.4 Results

4.4.1 Cytotoxicity

The HaCaT cytotoxicity (% cell density) of the whole honey (1.25% (v/v)) samples and protein fractions on the cell line was 99 ± 7% and 101 ± 7% for MA, 98 ± 7% and 103 ± 9% for BU, 97 ± 2% and 117 ± 7% for MF1, and ranged from 96 ± 6 - 104 ± 8% to 105 ± 13 - 115 ± 10% for all FB samples, respectively (Figure 4.4.1a and b). Differences between honeys were not significant and were only significant relative to the positive control, indicating the honeys and protein fractions were not cytotoxic at the concentration evaluated.

In the fibroblastic, SC-1 cell line, cytotoxicity (% cell density) of the whole honey samples at 1.25% (v/v) on the SC-1 cell line was 69 ± 6% for MA, 66 ± 4% for BU, 70 ± 10% for MA, and ranged from 61 ± 6 - 94 ± 15% for the FB samples (Figure 4.4.2a and b). As cytotoxicity was not desired for this study, a cell density <80% was not acceptable. Due to the cytotoxicity observed for FB2 and FB6, cytotoxicity was evaluated at a lower concentration of 0.625% (v/v). The % cell density was 116 ± 3% for MA, 114 ± 4% for BU, 110 ± 3% for MF1, and ranged from 104 ± 1 - 116 ± 4% for the FB samples and the differences compared with the control was not significant. The protein fractions had a cell density of 83 ± 4% for MA, 86 ± 1% for BU, 83 ± 7% for MF1, and ranged from 78 ± 1 - 86 ± 3% for the FB samples.

a



b

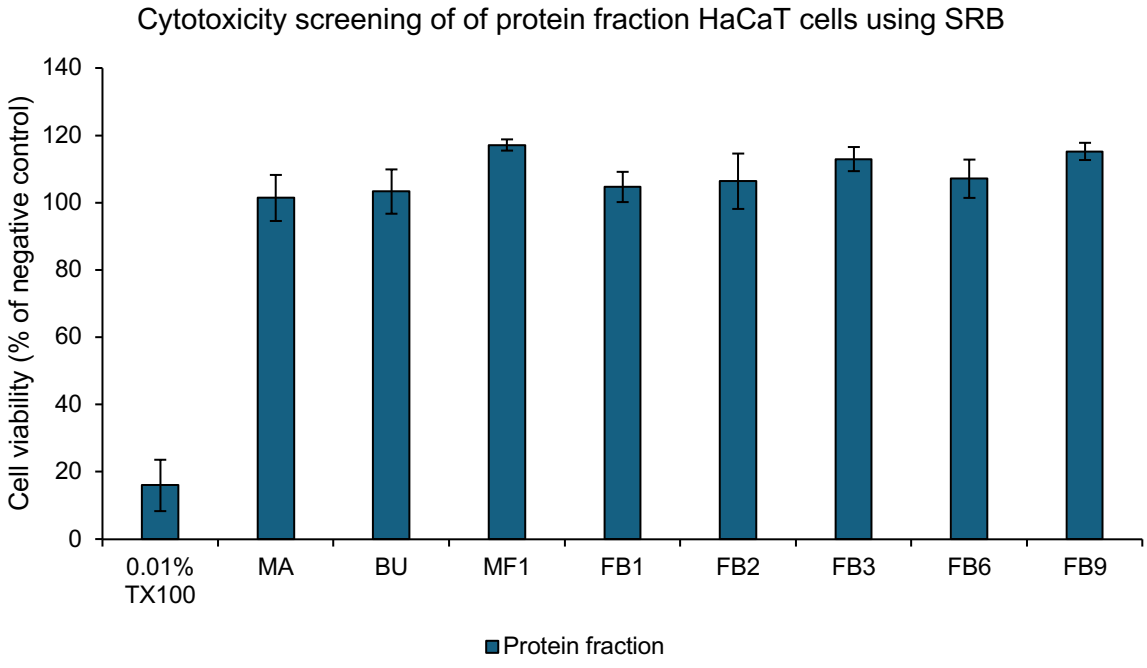
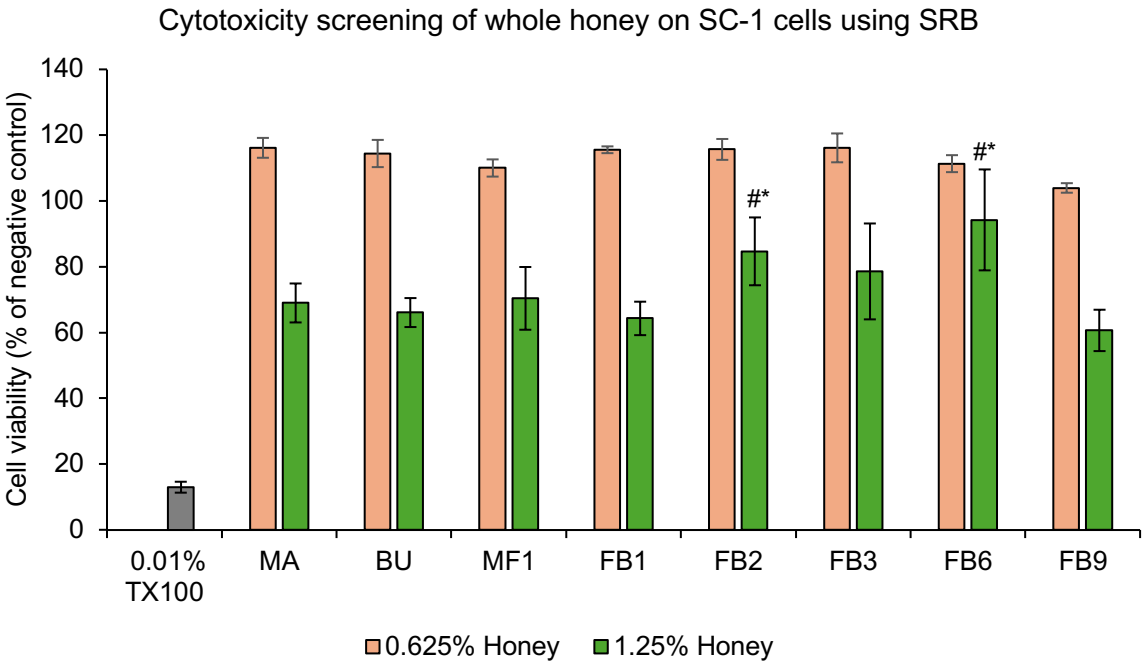


Figure 4.4.1. The cytotoxicity in HaCaT cells of (a) 1.25% (v/v) honey and the (b) 0.0044-0.0146 mg/g protein fraction analysed with the SRB assay. Data was expressed as percentage cell viability. Data is an average of three experiments ± SEM. Data is normally distributed and statistical significance was determined using ANOVA tests, $p \leq 0.05$. No statistical significance to MA and BU controls was detected.

a.



b.

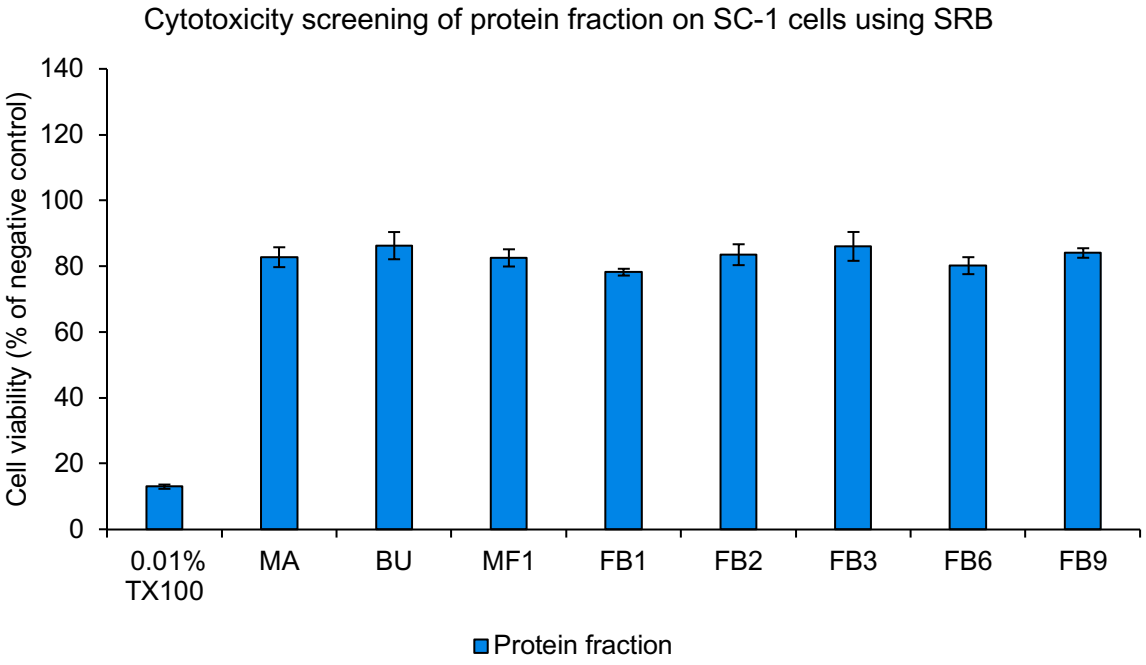
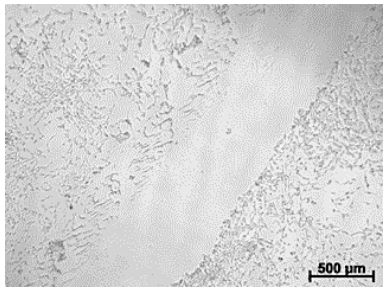
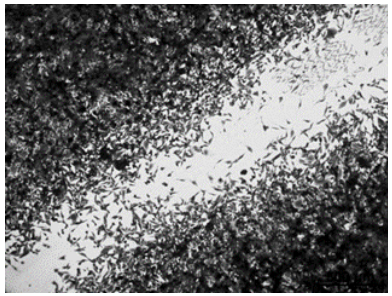
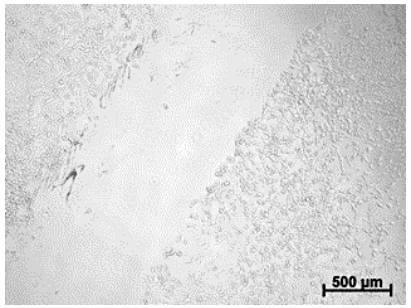
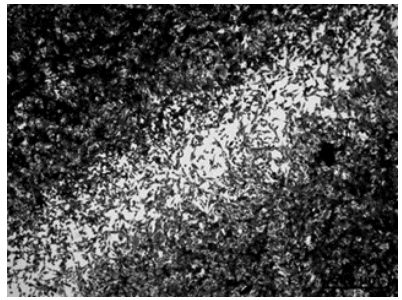
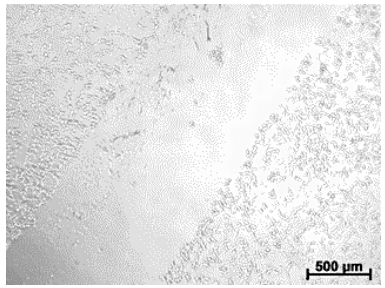
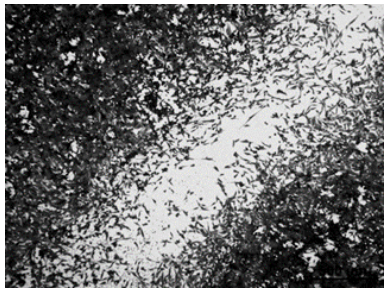
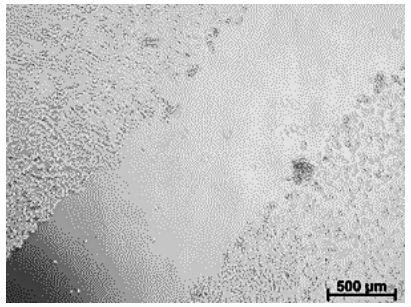
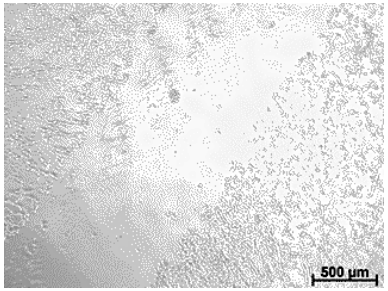
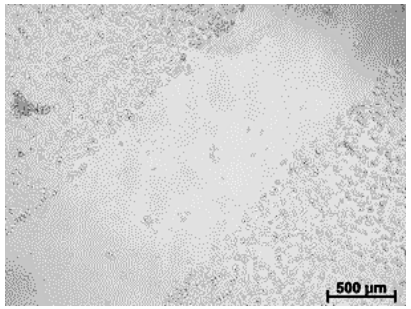


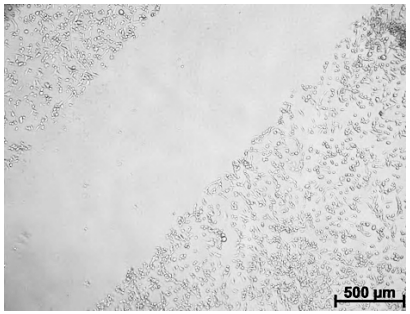
Figure 4.4.2. The cytotoxicity in SC-1 cells of (a) 1.25% and 0.625% (v/v) honey and the (b) 0.0044-0.0146 mg/g protein fraction analysed with the SRB assay. Data was expressed as percentage cell viability. Data is an average of three experiments ± SEM. Data is normally distributed and statistical significance was determined using ANOVA tests, $p \leq 0.05$. Samples denoted with # are significantly different to the MA whole honey control, and * are significantly different to the BU whole honey control.

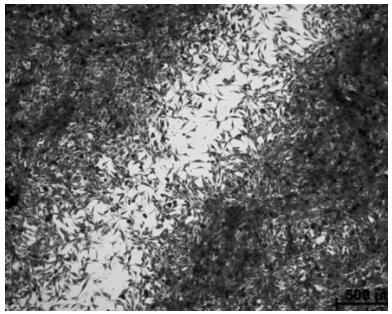
4.4.2 Cell migration

The scratch migration assay was used to determine the wound healing closure rate. Following the formation of the scratch wound, phase contrast microscopy was used to determine the scratch width at T0 and T24 at a 4x magnification before and following exposure to 2.5% FCS positive control, 0.01 M PBS vehicle/negative control and the honey samples and protein fractions. Figures 4.4.3 and 4.4.4 are representative images showing the differences in the scratch width at T_0 and T_{24} for both the HaCaT and SC-1 cell models (refer to Appendix B for all images). There was an observed increase in cell migration for all samples and controls after 24 h exposure and this was quantified.

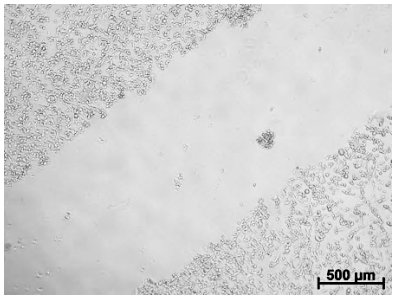
	T0	T24		T0	T24
PC			VC		
MA Honey			MA Protein		
BU Honey			BU Protein		

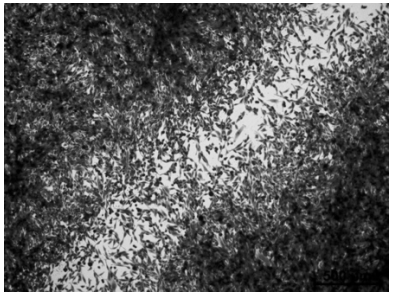
MF1 Honey



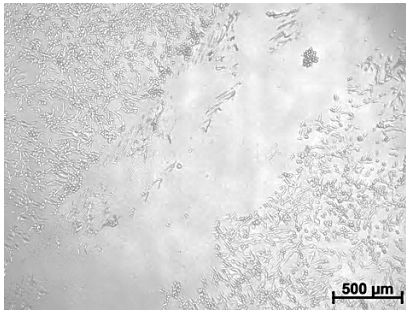


MF1 Protein

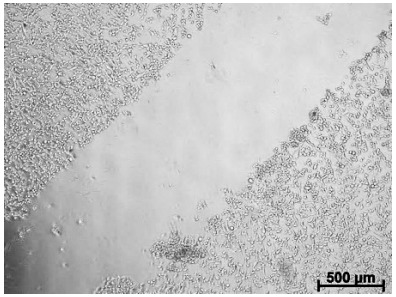




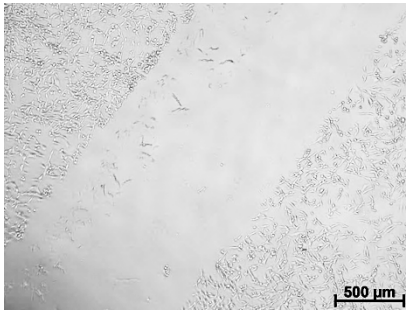
FB1 Honey

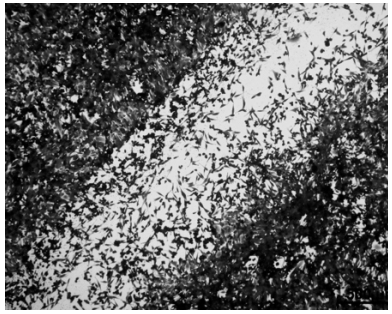


FB1 Protein

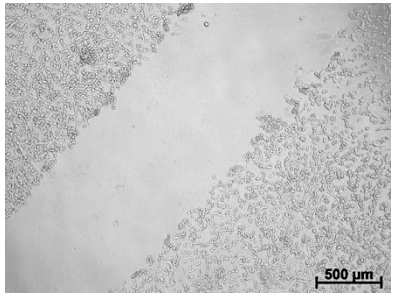


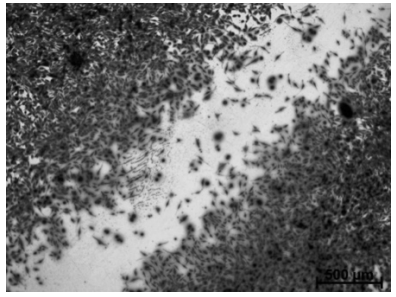
FB2 Honey





FB2 Protein





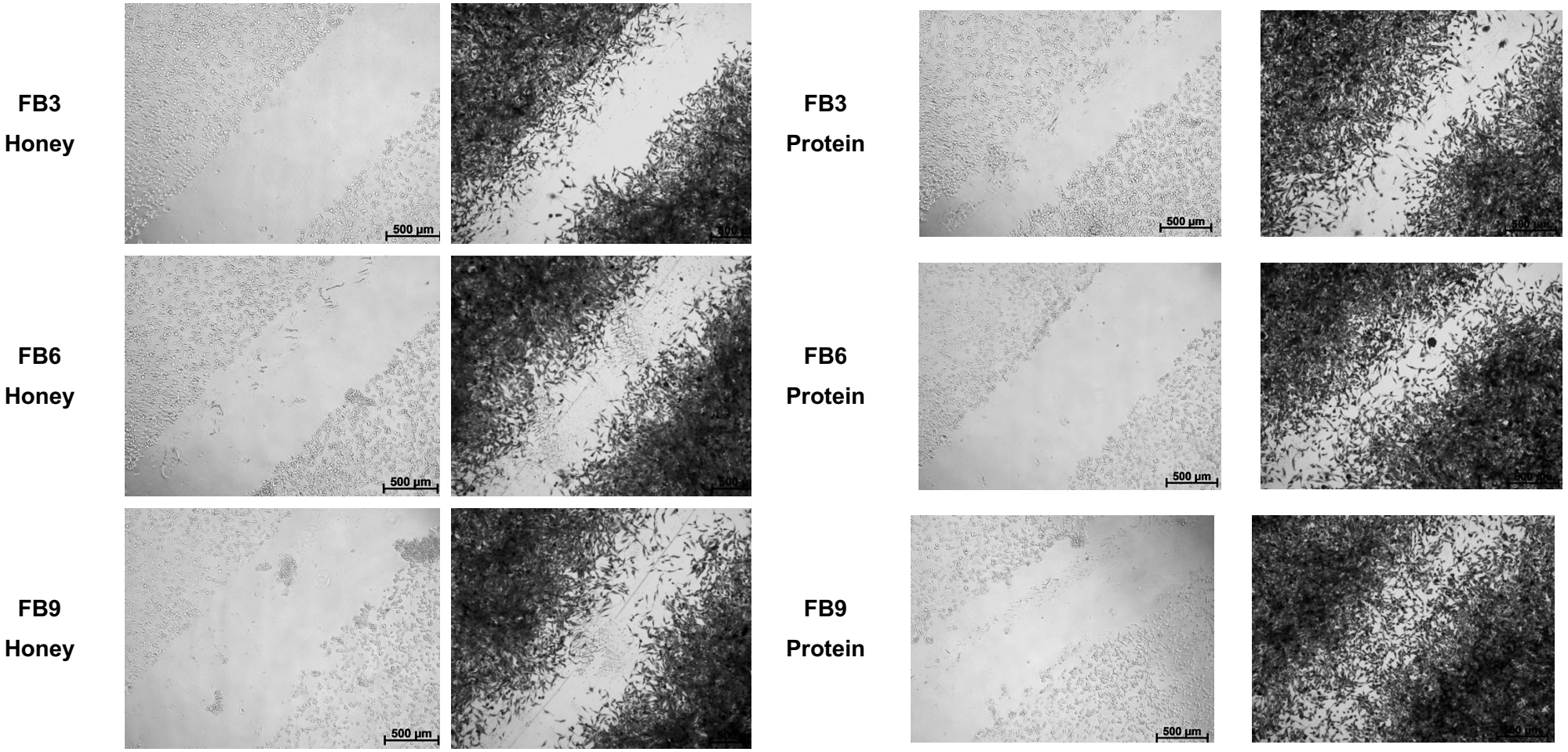
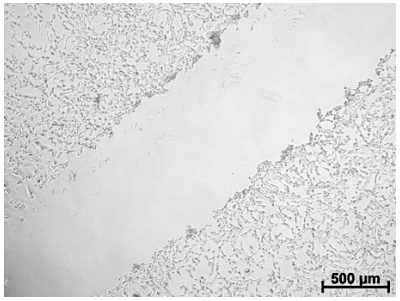
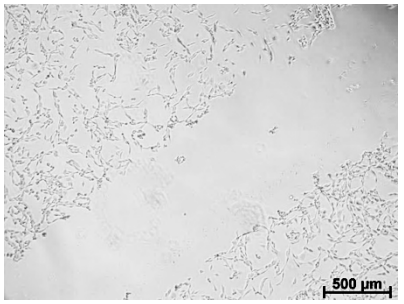
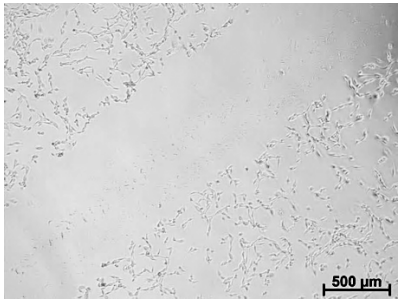
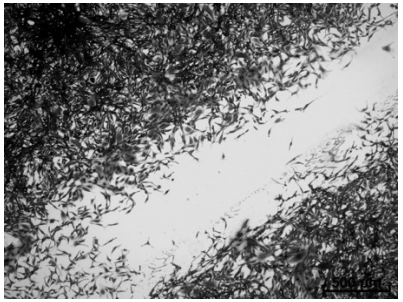
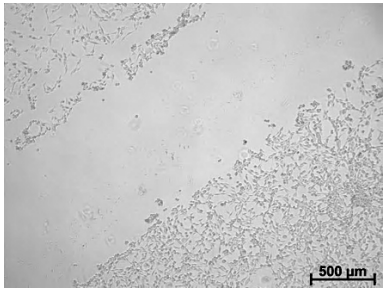
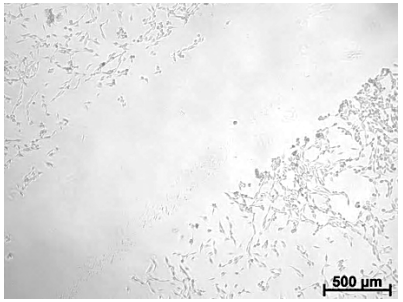
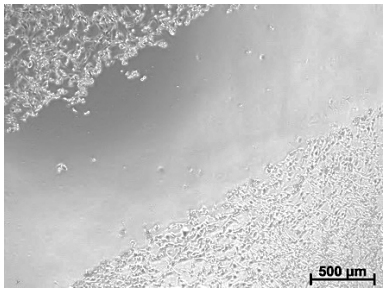
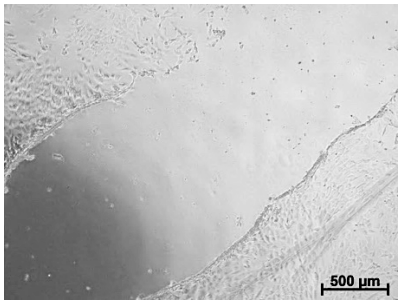
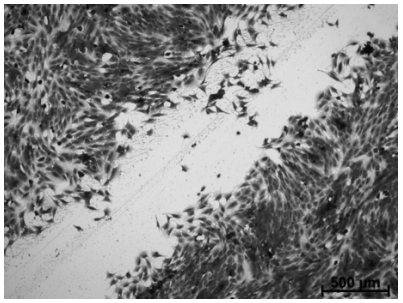


Figure 4.4.3. The HaCaT migration induced by 1.25% (v/v) honey and the 0.0044-0.0146 mg/g protein fraction assessed with the scratch assay. Images were taken 24 h apart, following CV staining, indicated as T0 (unstained cells) and T24 (CV stained cells). The positive control (PC) is 2.5% FCS and the vehicle/negative control (VC) is 0.01 M PBS Scale bar: 500 µm. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Data is expressed as representative of the duplicate data set for each sample (available in Appendix B). Images were analysed using the ImageJ software.

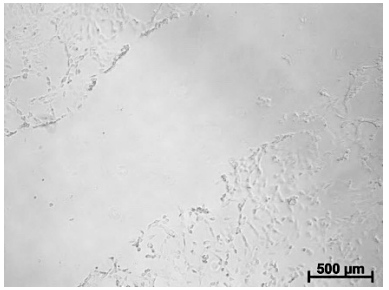
	T0	T24		T0	T24
PC			VC		
MA Honey			MA Protein		
BU Honey			BU Protein		

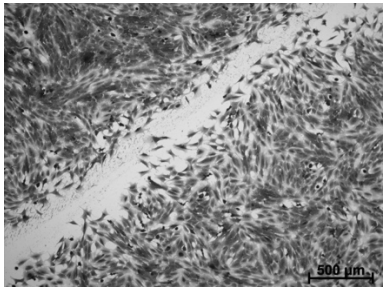
MF1 Honey



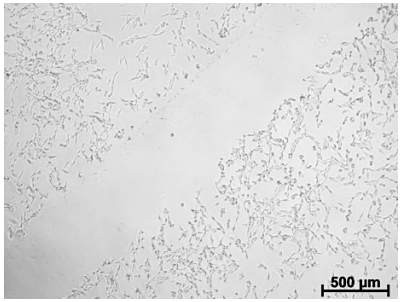


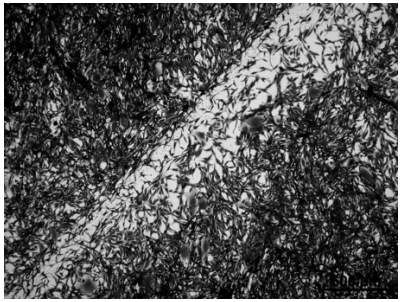
MF1 Protein



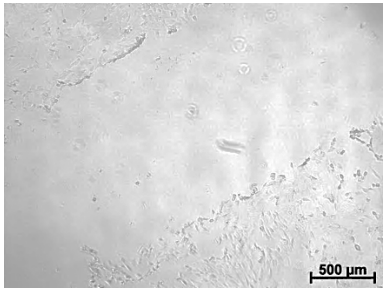


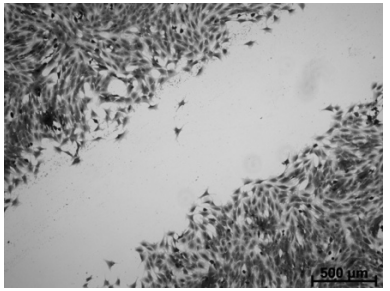
FB1 Honey



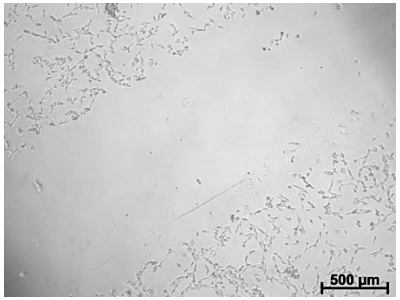


FB1 Protein

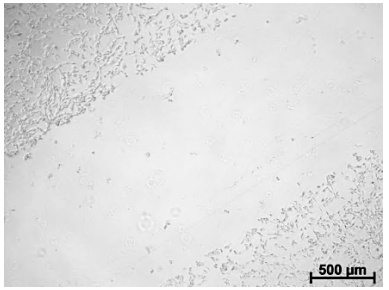




FB2 Honey



FB2 Protein



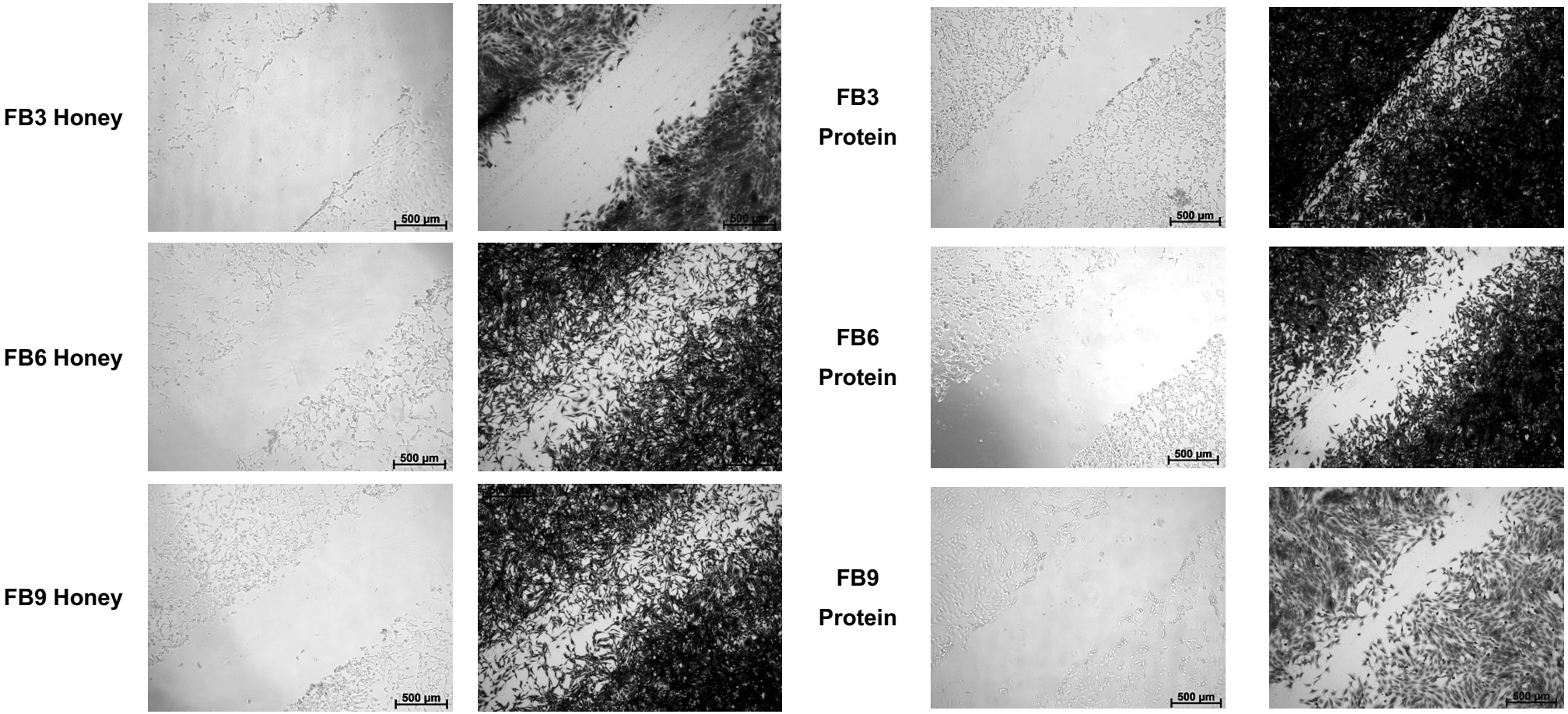


Figure 4.4.4. The SC-1 migration induced by 0.625% (v/v) honey and the 0.0044-0.0146 mg/g protein fraction assessed with the scratch assay. Images were taken 24 h apart, following CV staining, indicated as T0 (unstained cells) and T24 (CV stained cells). The positive control (PC) is 2.5% FCS and the vehicle/negative control (VC) is 0.01 M PBS Scale bar: 500 µm. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Data is expressed as representative of the duplicate data set for each sample (available in Appendix B). Images were analysed using the ImageJ software.

The HaCaT percentage wound closure rate for whole honey (1.25% (v/v)) was 84 ± 4% MA, 75 ± 9% for BU, 83 ± 6% for MF1, and ranged from 71 ± 8 - 90 ± 3% for all FB samples, respectively (Table 4.4.1). Honey samples FB1, FB2, and FB3 caused better wound closure than both the MA and BU controls, albeit samples MF1, FB6, and FB9 had improved wound closure compared to the BU control, although differences were not statistically significant.

The HaCaT percentage wound closure rate for protein (0.0044-0.0146 mg/g) samples was 76 ± 2% for MA, 82 ± 4% for BU, 74 ± 16% for MF1, and ranged from 75 ± 8 – 89 ± 3% for all FB samples, respectively (Table 4.4.1). Protein fractions FB1, FB2, and FB9 had improved HaCaT wound closure compared with the MA and BU protein fractions, whilst FB3 had an increased wound closure rate compared with the MA protein fraction, although not statistically significant. Honey sample FB2 and protein fraction FB9 induced the highest percentage wound closure rate. No significant differences in wound closure compared with 2.5% FCS (positive control) and MA indicates that these honeys and protein fractions potentially promote the migration of human keratinocytes in wounds.

For the honey (0.625% (v/v)), the percentage SC-1 wound closure rate was 44 ± 17% for MA, 48 ± 16% for BU, 76 ± 8% for MF1, and ranged from 46 ± 15 - 85 ± 3% for all FB samples, respectively (Table 4.4.1). Honey samples MF1, FB2, FB3, FB6, and FB9 caused better wound closure than both the MA and BU, albeit sample FB1 had improved wound closure compared with MA. No statistical differences, relative to 2.5% FCS (positive control), MA and BU honey was observed, indicating similar wound closure properties.

The percentage SC-1 wound closure rate for the protein fractions was 65 ± 7% for MA, 64 ± 4% for BU, 74 ± 7% for MF1, and ranged 66 ± 11 – 86 ± 6% for all FB samples, respectively (Table 4.4.1). Protein fractions MF1, FB1, FB2, FB3, FB6, and FB9 had improved wound closure compared with both the controls MA and BU. The protein fraction FB3 caused the greatest percentage wound closure. No statistical differences, relative to the 2.5% FCS (positive control), the MA and BU protein fractions was observed, indicating similar wound closure properties.

Table 4.4.1. The HaCaT and SC-1, wound closure (%) of honey and the protein fraction evaluated with the scratch assay.

		Wound closure (%)								
Sample type	**Cell line**	**PC (2.5% FCS)**	**MA**	**BU**	**MF1**	**FB1**	**FB2**	**FB3**	**FB6**	**FB9**
Honey	HaCaT (1.25% (v/v))	86 ± 5	84 ± 4	75 ± 9	83 ± 6	87 ± 3	90 ± 3	85 ± 7	78 ± 5	71 ± 8
	SC-1 (0.625% (v/v))	71± 8	44 ± 17	48 ± 16	76 ± 8	46 ± 15	65 ± 11	73 ± 9	75 ± 9	85 ± 3
Protein*	HaCaT	86 ± 5	76 ± 2	82 ± 4	74 ± 16	89 ± 2	84 ± 6	79 ± 6	75 ± 8	89 ± 3
	SC-1	71± 8	65 ± 7	64 ± 4	74 ± 7	66 ± 11	71± 10	86 ± 6	74 ± 8	72 ± 10

*The protein concentrations (final concentrations) were 0.0118 mg/g for MA, 0.0146 mg/g for BU, 0.0050 mg/g MF1, 0.0051 mg/g for FB1, 0.0068 mg/g for FB2, 0.0044 mg/g for FB3, 0.0126 mg/g for FB6, and 0.0123 mg/g for FB9

No significance was detected for the honey and protein fractions compared with 2.5% FCS positive control and Manuka controls, evaluated with GraphPad prism software version 9.5.0 (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com)

4.5 Discussion

Chemotherapeutic and cytotoxic drugs or infection hinders wound healing through a delay of cell migration at the wound site, decreasing collagen production, impeding fibroblast proliferation, preventing early stage matrix formation, and supressing wound contracture.[232] A non-cytotoxic concentration of the honey and the protein fraction was desired, therefore according to ISO 10993-5 cell viability of a compound of >80% is considered non-cytotoxic.[233] Therefore, the concentration of the honey and protein fractions was only accepted for further *in vitro* testing of bioactivity when the cell viability was >80% (Figure 4.4.1 and 4.4.2). The honey concentrations that fulfilled this requirement was 1.25% (v/v) for the keratinocyte (HaCaT) cells and 0.625% (v/v) for the murine fibroblast (SC-1) cells. The protein fractions did not induce cytotoxicity and further dilution was not required. The different sensitivity of the cell lines can be attributed to differences in genetics and phenotype that influence cellular responses to cytotoxic/cell migratory agents.[234] Furthermore, the doubling times for the HaCaT and SC-1 cell lines was 28 h[235] and 48 h[236] respectively. The doubling times of various cell lines can affect the interpretation of results generated in cell viability assays used to determine drug sensitivity or toxicity. Cell lines that proliferate faster than others are likely to have different metabolism or resistance profiles to a treatment compared to slower proliferating cells, complicating the assessment of treatment efficacy.[237] In addition, early apoptotic cells can be misinterpreted as viable resulting in misleading conclusions regarding cell viability.[238]

The cell viability (%) of the 1.25% (v/v) FB honey in HaCaT cells was 96 ± 6 – 104 ± 8% (Figure 4.4.1a) and 0.625% (v/v) FB honey in SC-1 cells was 104 ± 1 – 116 ± 4% (Figure 4.4.2a). A study by Ranzato *et. al.*[239] used 0.1% (v/v) Manuka and Buckwheat honey to treat a human fibroblast cell line (46 BR.1N) as this concentration was deemed suitable as it was below the EC_{05} of the Manuka and Buckwheat samples determined using the calcein-AM endpoint at 24 h.[239] According to Serem and Bester[152] the cell viability of Western Cape Fynbos honey on SC-1 cells at 0.8% v/v ranged between 90 - 110%, 75 - 110%, and 80 - 90% following 72 h exposure analysed using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), neutral red (NR), and CV assays respectively.[152] The cell viability for the same samples in SC-1 cells at 0.008 % v/v was between 100-110 %, 90-100 %, and 100-110 % following 72 h exposure analysed using the MTT, NR, and CV assays, respectively.[152] A study conducted by Yabes *et. al.*[240] used varying concentrations of Manuka honey at 40%, 60%, and 80% in human dermal keratinocytes (HEK-001), and following 24 h exposure the cell viability was 51%, 43%, and 27%, respectively.[240] Furthermore, Zekry *et. al.*[241] identified the cytotoxicity of a nanofibrous hydrogel treatment impregnated with a combination of 25% Manuka honey, 0.01% bee venom, and 25% lyophilised multiflora honey using the MTT assay on L929 mouse

fibroblast cells. It was observed that the cell viability was inversely proportional to honey concentration.

While *in vitro* cytotoxicity analysis is crucial, limitations do exist, for example these cell cultures are representative of a single cell type, and consequently the extrapolation to *in vivo* testing is often poor. Consequently, a combination of *in vitro* and *in vivo* analysis is essential in comprehensive screening of safety and efficacy.[242] Furthermore, the SRB, CV, MTT, and NR assays have different principles. The SRB assay dye binds to basic amino acid residues under slightly acid conditions,[226] and the CV assay Positively charged dye that binds to negatively charged cellular proteins and DNA.[243] Conversely, the MTT assay is a metabolic assay based on the cleavage of the tetrazolium ring of MTT (3-(4,5-dimethylthazolk-2-yl)-2,5-diphenyl tetrazolium bromide) by dehydrogenases in active mitochondria of living cells as an estimate of viable cell number.[244] Finally, the NR assay uses the lysosomes of viable cells, lysosomes maintain an acidic environment (~4.5–5.5 pH) through vacuolar ATPase (V-ATPase) proton pumps, causing the pH-sensitive neutral red dye to become protonated and accumulate, with greater accumulation indicating a higher number of viable cells.[245] The choice of cell viability assay can affect the comparability of results, as each method evaluates a different aspect of cell health. This variation can lead to inconsistencies in data interpretation, particularly when comparing studies that use different assays. For instance, one assay may be more sensitive to metabolic activity, while another focuses cellular protein or DNA presence, potentially producing different viability estimates for the same treatment condition.[246]

The HaCaT wound closure rate (%) for 1.25% (v/v) FB honey was 71 ± 8 – 90 ± 3% for the Fynbos samples (Figure 4.4.3). A study conducted by Martinotti *et. al.*[247] analysed the effect of 0.1% Manuka honey on HaCaT cells indicating an ± 90% wound closure after 24 hrs compared with the 2.5% FCS positive control. That wound closure rate is higher than what was generally observed in the present study but is comparable to the 90 ± 3% wound closure for FB2 honey sample (Table 4.4.1).

The SC-1 wound closure rate (%) for 0.625% (v/v) FB honey was 46 ± 15 – 85 ± 3% (Figure 4.4.3). Chaudhary *et. al.*[220] reported that primary fibroblast cells exposed to 0.1% Manuka honey exhibited a wound closure rate of 60% to 70% after 24 h. The percentage for SC-1 wound closure for FB2 honey was similar or greater for MF1, FB3, FB6, and FB9 honey, than reported by Chaudhary *et. al.*[220] Sell *et. al.*[221] identified the *in vitro* migratory effects of 1% (v/v) Manuka honey in human fibroblast cells (HDF) evaluated at 6 h, 12 h, 18 h, and 30 h. Wound closure was 19%, 55%, 70%, and 90%, respectively. The HaCaT wound closure rate

(%) for the protein fractions on the cell line was 75 ± 8 – 89 ± 3% for all the Fynbos samples, (Figure 4.4.3 and Table 4.4.1). The SC-1 wound closure rate (%) for the protein fractions was 66 ± 11 – 86 ± 6 for all FB samples (Figure 4.4.3 and Table 4.4.1). The wound closure rate of the honey samples at 1.25% (v/v) (HaCaT cell model), 0.625% (v/v) (SC-1 cell model) and protein samples (HaCaT and SC-1 cell models) was fairly stable and consistent in both cell lines. Indicating a potential migratory activity of the proteins found in honey as that activity is not inhibited following isolation.

It has been reported that honey can activate cyclin-dependent kinase 2, focal adhesion kinase, and rasGAP SH3 binding protein 1, crucial role players in regulation cell proliferation, migration and .[222] Cyclin-dependent kinase 2 is a key regulator of the cell cycle, particularly the G1 to S phase transition, facilitating keratinocyte and fibroblast proliferation, which are essential for re-epithelialization and tissue remodelling.[248] Focal adhesion kinase is a non-receptor tyrosine kinase that promotes cell migration, adhesion, and survival through interactions with integrins and the actin cytoskeleton.[249] It also enhances angiogenesis by stimulating endothelial cell motility and proliferation while supporting fibroblast-mediated ECM deposition and wound contraction.[249] Meanwhile, rasGAP SH3 binding protein 1 contributes to wound healing through its role in Ras signalling, which regulates cell survival and proliferation.[250] Additionally, it influences fibroblast activity in ECM remodelling and helps mitigate oxidative stress, preventing further tissue damage. [250] Together, these proteins coordinate essential processes in wound repair, ensuring effective tissue regeneration. Ranzato *et. al.*[222] identified the ability of Manuka, Buckwheat, and Acacia honeys to up-regulate or down-regulate regulatory genes in epithelial-mesenchymal transition and re-epithelialisation thereby promoting keratinocyte migration.[222]

Moreover, the antioxidant capacity, that reduces oxidative stress, can further provide an environment that favours cellular migration at the wound site.[244] Although the reduction in oxidative stress has been previously linked to improved cell migration, the % oxidative damage of the honey and protein samples ranged between 82.2 ± 4.4 – 100.0 ± 8.2% and 96.6 ± 5.1 – 117.1 ± 7.3% for the HaCaT cell model and 79.1 ± 3.1 – 96.0 ± 3.3 % and 82.2 ± 6.7 – 95.0 ± 8.5% for the SC-1 cells cell model, respectively (refer to table 5.4.1). Therefore, the high % of oxidative damage indicates a low cyto-protection against oxidative stress, thus, suggesting the stabilised wound closure rates for both the honey and protein samples on both cell models is likely due to MMP-9 stimulation DF-1.

A study conducted by Bucekova *et. al.*[139] identified a dose dependent relationship between DF-1 and MMP-9 secretion that promoted keratinocyte migration *in vitro*.[139] MMP-9 is a protease responsible for the detachment of keratinocytes from the basement membrane facilitating keratinocyte migration and ultimately wound re-epithelialisation. Therefore, this dose dependent relationship identified by Bucekova *et. al.*[139] could explain the observed stabilisation of the wound closure rates of the protein isolates, as the protein samples do not experience a significant loss of wound closure activity in comparison to their whole honey counterparts. Furthermore, the presence of a ±5.52 kDa band[245] identified using SDS-PAGE found within the protein isolates (chapter 3, figure 3.4.6), hypothesised to be DF-1, could explain this stabilisation in activity. However, this is not an exact indication of DF-1 presence further investigation using LC-MS/MS and MALDI-TOF analysis is needed to further fingerprint the protein and honey samples.

4.6 Conclusion

The cell viability for the honeys at 1.25% (v/v) (HaCaT cell model) and 0.625% (v/v) (SC-1 cell model) and the protein fraction in both cell lines was greater than 80% indicating no significant toxicity at these concentrations. These concentrations were then used for further *in vitro* studies. The wound closure rate of the honey samples at 1.25% (v/v) (HaCaT cell model), 0.625% (v/v) (SC-1 cell model) and protein samples (HaCaT and SC-1 cell models) was stabilised in the protein samples across both cell lines. This indicates a potential relationship between cell migration and the presence of DF-1, although tentatively identified in chapter 3, this would need to be confirmed with further LC-MS/MS analysis.

Chapter 5: The antioxidant and anti-inflammatory activity of selected South African honeys and protein extracts

5.1 Introduction

Antioxidants are compounds that decrease the cellular damage caused by ROS through the reduction of oxidative stress and protecting antioxidant enzymes by neutralising free radicals.[246] Honey is an ideal natural antioxidant as it contains both lipophilic and lipophobic antioxidants allowing for activity at multiple cellular levels. These antioxidants include vitamin A (retinol), carotenoids, vitamin C (ascorbic acid), monophenols, polyphenols, and flavonoids.[114] In wounds, antioxidants counteract exaggerated tissue deterioration and damage caused by O_2, OH, superoxide, H_2O_2, or lipid peroxyl radical oxidant.[46] Beyond their role in counteracting oxidative stress, antioxidants also influence key aspects of the inflammatory response, which is closely linked to wound healing outcomes.

The local inflammatory response surrounding the wound site influences the composition and volume of wound exudates. Consequently, honeys anti-inflammatory properties mitigate oedema and exudates, thereby enhancing wound healing.[114] This reduction of oedema and inflammation directly alleviates pain resulting from pressure on the nerve endings and reduces the production of prostaglandins during the inflammatory response.[114] Therefore the investigation of the antioxidant and anti-inflammatory activity of the honey samples and the protein fraction, is valuable in determining the potential wound healing effects. Pro-oxidants are agents that generate ROS, are oxidising agents responsible for cell damage but are often beneficial in the normal wound healing response of acute wounds.[247] A balance between high and low levels of ROS is essential, low concentrations beneficially protect the wound site from infection and initiate effective wound healing.[247,248] Regulated through cellular signally pathways such as, NF-kB responsible for fibroblast migration and proliferation crucial for downstream collagen synthesis and subsequent wound contracture.[247,248]

Cellular and acellular models are combined when investigating the bioactivity of a nutraceutical as both offer complementary insights into the mechanism of action. Cellular models allow the assessment of the effect of compounds on cellular function including proliferation or enzymatic activity.[249] Conversely, acellular models, like biological experiments in simulated body fluids provides insights into an isolated or single aspect of the interaction of a compound with the environment. However, acellular models lack the complexity that cellular models offer.[250] A hybrid approach integrates cell morphology and chemical structure data to improve the accuracy of bioactivity prediction. Therefore, researchers can achieve a detailed

understanding of both the physiochemical properties and biological impact of the compounds leading to a thorough assessment of bioactivity and applications across diverse fields.[249,250]

Integration of the chemical oxygen radical absorbance capacity (ORAC) and trolox equivalent antioxidant capacity (TEAC), and the cellular 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assays provides a more complete evaluation of different aspects of antioxidant activity.[251] The TEAC assesses the scavenging of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) radicals and is an electron transfer assay, while the ORAC assay which is considered more physiologically relevant evaluates antioxidant activity against peroxyl radicals. The principle of the DCFH-DA is similar to the ORAC assay, except that antioxidant activity is evaluated within a cellular environment using a relevant cell line.[252] This holistic approach allows researchers to obtain a thorough understanding of the antioxidant capabilities of various samples, their potential health benefits, and practical applications.[253]

Honey has been known to exhibit significant anti- and pro-inflammatory activity, contributing to its therapeutic potential in inflammatory conditions. Manuka honey has shown significant reduction in pro-inflammatory cytokines (IL-8) in patients with gingivitis identified by Al-Kubaisi *et. al*.[254] According to Mohammed255 the flavonoids within honey are known to inhibit the pro-inflammatory enzyme (COX) and transcription factors (NF-kB), reducing inflammatory responses responsible for significant severity in sores in patients with eczema and psoriasis.[255] Wu *et. al.*[256] identified a link between the anti-inflammatory properties of honey and modulation of the gut microbiome in geriatric patients using an *ex vivo* gut model.[256] Furthermore, a study done by Tonks *et. al.*[257] identified a 5.8 kDa component of Manuka honey responsible for stimulation of TNF- α immune cells via the toll like receptor (TLR)-4 pathway.[257] A study conducted by Owoyele *et. al*.[258] investigated the effect of honey on NO production in Wistar rats, where honey demonstrated an ability to inhibit NO release (anti-inflammatory effect) suggesting a complex dual interaction of honey with either an increase or decrease of NO.[258] While these properties have been primarily attributed to the phenolic acids and flavonoids, it is worth considering whether the proteins within honey, an overlooked component, also contribute may to this anti-inflammatory activity.

The pro- or anti-inflammatory activity of samples such as honey can be assessed with the an integrated approach, where with the sodium nitroprusside (SNP) assay, the ability of samples to directly scavenge nitric oxide (NO) while in the RAW 264.7 cells model not only LPS-induced NO scavenging can be assessed but the ability of samples to induce NO can also be

evaluated.[259] In addition, inflammatory markers associated with either process can also be evaluated.[259]

Therefore, the aim of the research undertaken in this chapter is to evaluate the antioxidant and the pro- and anti-inflammatory properties of FB honey and the protein fractions compared with Manuka and Buckwheat honey.

The specific objectives for this chapter were:

1. To determine, with the TEAC, ORAC and CAA assay, the chemical and cellular antioxidant activity of each honey and their respective protein fractions.
2. To determine using the LPS-induced NO/RAW 264.7 cell model the anti-inflammatory activity of each honey and their respective protein fractions.

5.2 Materials

Honey samples

The honey samples and protein fractions used in the previous chapters will be used in this study.

Reagents, equipment and disposable plasticware

Reagents used are the same as in Chapters 3 and 4 and in addition: Fluorescein, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), ABTS, 35% (m/m) formaldehyde, $K_2S_2O_8$, DCFH-DA, (±)-6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (Trolox), LPS from *E. coli* O111:B4 (Sigma Aldrich, Atlasville, SA), Na_2HPO_4 (Merck, Modderfontein, SA), $NaH_2PO_4.H_2O$, sulphanilamide, N-1-naphthylethylenediamine (NED) (Riedel-deHaën, Honeywell Research Chemicals, Midrand, SA), Sodium nitroprusside (SNP) (Analar analytical reagent, Hopkins & Williams LTD, London, UK were used.

All equipment and disposable plasticware is the same as used previously, except the cell culture treated polystyrene 96 well plates in clear and opaque white, which were obtained from Greiner Bio-one supplied by Lasec, Johannesburg, SA.

Cell lines

Cell lines were the same as used previously. RAW 264.7 cell line was obtained from ATTC, The Global Bioresource Centre, Virginia, USA and were used at passages 18-22.

5.3 Methods

Trolox equivalent antioxidant capacity assay

In the TEAC assay, via an electron transfer mechanism, the antioxidant is oxidised, while the oxidant probe is concurrently reduced. As the oxidant probe undergoes reduction, its colour intensity decreases, providing a measure directly linked to the concentration of the antioxidant being analysed.[260]

For this assay a stock solution of ABTS was prepared by dissolving 4.00 mg $K_2S_2O_8$ and 21.9 mg ABTS in 5 mL of 0.01 M PBS to obtain a final concentration of 3 mM $K_2S_2O_8$ and 8 mM ABTS. This stock solution was incubated for ± 12h in the dark at room temperature and was then further diluted 30X in 0.1 M PBS. A standard curve, with Trolox, 0 - 300 µM diluted in 0.01 M PBS was prepared. To 10 µL each of the different Trolox concentrations, the 25% (v/v) honey solutions or the individual protein fractions, 290 µL of the 30X diluted ABTS solution was added. After 10 minutes at room temperature in the dark, the colour intensity was measured at 734 nm using the FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany).[260] Using the standard curve ($r^2 > 0.95$) the concentrations were determined and were expressed as µM TE per gram (TE/g), after the weight of the honey samples and protein fractions was considered.

Oxygen radical absorbance capacity assay

In the ORAC assay, a hydrogen atom transfer mechanism is employed. The antioxidant donates a hydrogen atom to the free radical in the same manner as a substrate, which in the ORAC assay is fluorescein Both the antioxidant and substrate compete for free radicals. Therefore, a strong antioxidant will quench the radials by donating hydrogen atoms to the free radicals at a faster rate than the probe substrate will bind to free radicals, with a resultant smaller decrease in fluorescent intensity of the probe.[261,262]

For this assay, a 0.139 nM solution of fluorescein solution was prepared by dissolving 3,32 mg of fluorescein in 10 mL of ORAC PBS (3.87 M Na_2HPO_4 and 6.12 M $NaH_2PO_4.H_2O$, diluted in 1L of ddH_2O, pH 7.0). A 10 µL aliquot of this fluorescein solution was further diluted to 10 mL with ORAC PBS and was labelled F1. F1 was further diluted, by diluting 100 µL, to a final volume 10 mL in ORAC PBS to prepare the working solution, F2. All fluorescein solutions are light sensitive and therefore were covered in tin foil and kept in a dark environment. A 2 mL solution of 0.24 M AAPH (65.0 mg in 1 mL of ddH_2O) and a 2 mL solution of 1 mM Trolox solution (2.5 mg dissolved in 10 mL, 0.01 M PBS) was prepared. The 1 mM Trolox solution was used to make a concentration range of 0 – 300 µM. A 10 µL volume of each Trolox

concentration, honey, or protein fraction were added to individual wells of a white opaque 96 well plate. The control was 165 µL of the F2 solution followed by the addition of 25 µL of the 0.24 M AAPH solution. Equivalent volumes of 0.01M PBS, with and without AAPH served as the blanks.[261,262] The fluorescence was measured using the FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany), at an excitation wavelength of 485 nm and an emission wavelength of 520 nm, every minute for 2 h. The instrument settings were shaking at 500 rpm; temperature was 37°C; and gain adjustment with 200 µL fluorescein only (maintained between 900 – 950). For each sample the area under the curve was calculated and from the Trolox standard curve (r^2 > 0.95) the antioxidant activity of the honey and protein fractions was determined, expressed as µM TE/g,[261,262] after the weight of the honey samples and protein fractions was considered.

Cellular 7'-dichlorodihydrofluorescein diacetate assay

The human keratinocyte (HaCaT) cells and murine fibroblasts (SC-1) were plated under sterile conditions at $10x10^4$ cells/mL in white opaque, clear bottom, 96 well plates and incubated overnight at 37°C and 5% CO_2, for attachment. Adherent HaCaT cells were exposed to 10 µL of 12.5% (v/v) (final concentration, 1.25% (v/v)) honey and SC-1 adherent cells were exposed to 10 µL of 6.25% (v/v) (final concentration, 0.625% (v/v)) honey. Both cell lines were treated with the protein fractions (concentration range 0.044 - 0.118 mg/g). To determine pro-oxidant activity no AAPH was added while for the determination of antioxidant activity 10 µL of 40 mg/mL AAPH (final concentration, 4 mg/mL) was added. Controls were 10 µL 0.01 M PBS with 10 µL, 40 mg/mL AAPH and 20 µL 0.01 M PBS only.[263]

After a 48 h exposure, at 37°C and 5% CO_2, DCFH-DA was added. A 750 µM stock DCFH-DA solution was prepared in 0.01 M PBS, pH 7.4 which was further diluted 10x to a working solution of 75 µM. A 50 µL volume of working solution was solution was added to each well and incubated for 1h at 37°C and 5% CO_2. The change in fluorescence was then measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). The positive control was used to adjust the instrument gain parameter. All data was expressed relative to the AAPH positive control, 100% oxidate damage. The percentage oxidative damage calculated as follows:

Oxidative damage (%) = [(Sample - Negative control)/(Positive control - Negative control)]*100[263]

Direct NO scavenging

Nitric oxide in excess has a pro-inflammatory effect. The reduction of NO by bioactive molecules reduces inflammation and promotes wound healing. Sodium nitroprusside (SNP) spontaneously generates NO at physiological pH, this NO competes with oxygen molecules forming either nitrites or nitrates, which are stable metabolites of NO.[18] NO has a very short half-life, therefore measuring the nitrite or nitrate content is a more reliable method of quantifying NO. These stable NO metabolites can be used to quantify the scavenging ability of a treatment, subsequently quantifying the anti-inflammatory activity.[264] Greiss reagent is used to quantify the nitrite/nitrate concentration through the interaction of these ions with sulphanilamide forming a diazonium salt.[265,266] This salt reacts with N-(1-naphthyl)ethylenediamine (NED) forming a pink/red azo dye whose absorbance values can be quantified, these absorbance units are directly proportional to the concentration of nitrite/nitrate ions.[265,266]

A 5 mM solution of SNP (15 mg in 10 mL) was prepared and left to incubate at room temperature in the light for 1.5 h. A 50 µL volume of 25% (v/v) honey and the protein fractions were added to the wells of a 96 well plate. The same volume of 0.1 M PBS and ddH_2O served as blanks. Then 50 µL of the activated 5 mM SNP solution in 0.1 M PBS was added and incubated for 1 h in the dark at room temperature. The NO formed was quantified with the Griess reagent consisting of 0.1 g sulphanilamide and 0.01 g of NED dissolved in 10 mL of a 2.5% H_3PO_4 solution, generating a 1% (w/v) sulphanilamide and 0.1% (w/v) NED solution. For quantification 50 µL of Griess reagent was added to each well and the 96 well plate was gently mixed. The 96 well plate was then read at 550 nm using the Synergy II spectrophotometer (Biotek Instruments, Inc., Winooski, USA). The NO scavenging activity of the honey samples was expressed as a percentage of the SNP (no sample added, 100% NO produced).[264]

Lipopolysaccharide induced RAW 264.7 cell model

Lipopolysaccharide (LPS) is a component found in the cell walls of Gram-negative bacteria that induces NO formation and the activation of inflammatory pathways in the RAW 264.7 macrophage cell line. LPS binds to TLR's on the macrophage cells, specifically TLR4, subsequently activating NF-κB transcription factor initiating the transcription of cytokine genes, producing pro-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-8, and platelet-activating factor).[267,268] The activation of NF-κB and downstream production of inflammatory cytokines leads to the expression of iNOS which is an responsible for NO production in immune cells.[269,270] Therefore the cytokine environment of the macrophage cells can determine the

rate of NO production.[271] The ability of bioactive treatments to reduce NO levels can be measured with the Griess assay.

A volume 80 µL of RAW 264.7 cells were plated at a density of 1.25 x 10^6 cells/mL (final concentration 1 x 10^6 cells/mL) in the wells of a clear 96 well plate. A 10 µL volume of the 12.5% (v/v) honey or protein fractions together with 10 µL of a 1000 ng/mL LPS solution (final concentration 100 ng/mL) was added to each well. The positive control was RAW 264.7 cells exposed to 10 µL of LPS (1000 ng/mL) and 10 µL, 0.01 M sterile PBS, and the negative control was RAW 264.7 cells exposed to 20 µL, 0.01 M sterile PBS. The cells were then incubated for 24 h at 37°C and 5% CO_2. A volume of 50 µL of the cell supernatant from each triplicate well was transferred to another 96 well plate and 50 µL Griess reagent was added. The absorbance was then measured at 570 nm using the Epoch spectrophotometer (Biotek Instruments, Inc., Winooski, USA). For this assay a sodium nitrite ($NaNO_2$) standard curve of 0 –100 nM was prepared ($r^2 > 0.95$) and the data was reported as mM $NaNO_2$.[272,273] The remaining 50 µL of medium and adherent cells were used to determine cell viability using the CV assay.

CV staining

A volume of 5 µL of 20% v/v formaldehyde (final concentration, 2% v/v) was added to each well and was left at 37°C for 30 minutes. The well contents were discarded, the plate was washed using tap water and left to dry. Then 100 µL of 0.1% (w/v) CV dye (prepared in 0.75% (v/v) formic acid at pH 3.5) was added to each well allowing the attached cells to stain at room temperature for 30 minutes. The dye solution was discarded, the plate was washed with tap water and left to dry. The dye was then extracted with 100 µL of 10% (v/v) acetic acid on the plate shaker for 30 minutes. The absorbance was measured using the BioTek Epoch spectrophotometer at 630 nm. Data was expressed as % cell viability relative to the control.[274]

Data management and statistical analysis

Data was an average of three experiments where each measurement was done in triplicate, subsequently, 9 data points were generated per sample (independent variable) treated as dependent variables. All data was expressed as mean ± SEM of the triplicate experiments analysed using Microsoft 365 Excel 2023. Determination of parametric and non-parametric data was conducted using the D'Agostino and Pearson test; and Shapiro-Wilk test using the GraphPad prism software version 9.5.0 (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Data was statistically analysed using one-way ANOVA and Tukey's multiple comparisons test was conducted for comparison of means using the same statistical

software. Pearson coefficients were used to measure the association between two normally distributed variables.

5.4 Results

5.4.1 Antioxidant activity

Determined with the TEAC assay (Figure 5.4.1), the antioxidant capacity for the honey samples was 4.62 ± 0.16 µM TE/g for MA, 8.66 ± 0.14 µM TE/g for BU, 3.27 ± 0.31 µM TE/g for MF1, and ranged from 2.05 ± 0.35 - 6.27 ± 0.46 µM TE/g for the FB samples. The honey control BU had the greatest antioxidant capacity, and the Fynbos honey with the highest activity was FB6, both with antioxidant activity significantly greater than MA. The antioxidant capacity of all the protein fractions were low and was 0.19 ± 0.06 µM TE/g for MA, 0.33 ± 0.08 µM TE/g for BU, 0.15 ± 0.02 µM TE/g for MF1, and ranged from 0.12 ± 0.05 - 0.23 ± 0.06 µM TE/g for the FB samples and differences were not statistically different. A 90-98% decrease in the antioxidant capacity for the protein fractions indicates that these fractions do not contribute significantly to antioxidant activity evaluated with the TEAC assay.

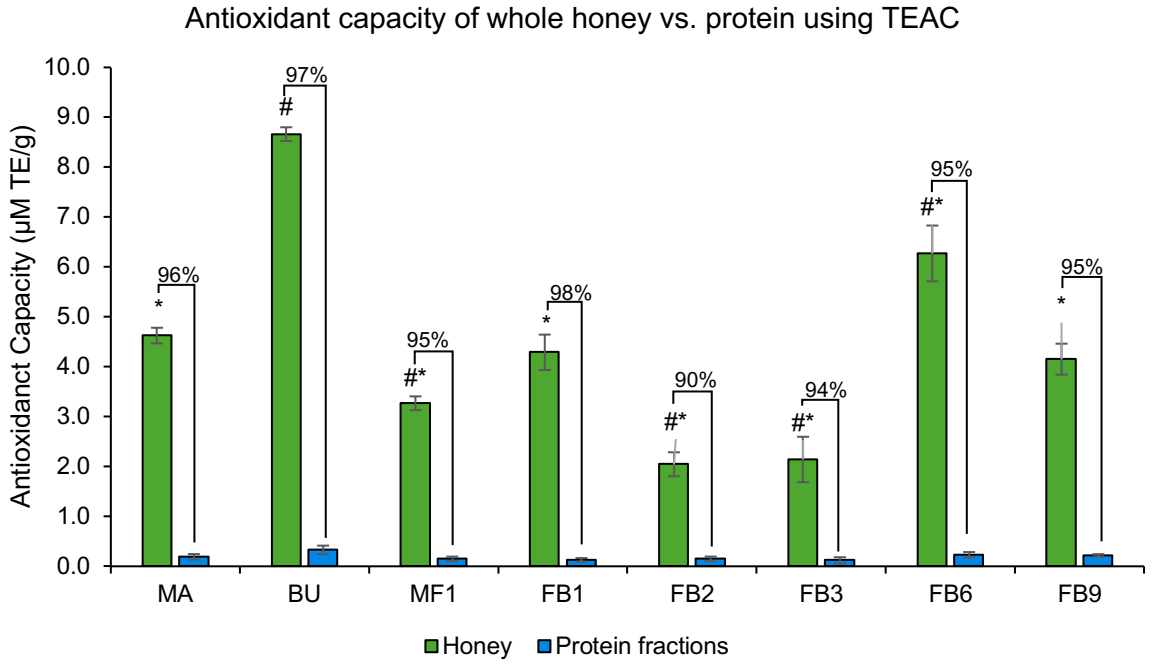


Figure 5.4.1. Antioxidant activity, TEAC assay of the honey samples and protein fractions. Data was expressed as Trolox equivalent (TE) µM TE/g. Data is an average of three experiments ± SEM. Data is normally distributed, and statistical significance was determined using ANOVA tests. Samples denoted with # are significantly different to the MA whole honey control, and * are significantly different to the BU whole honey control, p≤0.05.

The antioxidant capacity, determined with the ORAC assay (Figure 5.4.2) for the different honeys was 1.88 ± 0.31 µM TE/g for MA, 1.55 ± 0.40 µM TE/g for BU, 2.10 ± 0.47 µM TE/g for MF1, and the range was 1.79 ± 0.41 - 2.67 ± 0.73 µM TE/g for the FB honeys. Honey sample FB9 had the greatest antioxidant capacity, significantly greater than the BU control sample. All other Fynbos honey samples (FB1-FB6) had an antioxidant capacity similar to BU and MA, as no statistically significant differences were found.

The antioxidant capacity for the protein fractions was 0.06 ± 0.01 µM TE/g for MA, 0.09 ± 0.02 µM TE/g for BU, 0.03 ± 0.02 µM TE/g for MF1, and ranged from 0.03 ± 0.01 - 0.09 ± 0.01 µM TE/g for the FB samples. Honeys MF1, FB, FB2 and FB3 had significantly less activity than BU while the antioxidant activity of FB6 and FB9 was similar to MA and BU. A 94-99% reduction in activity compared with the honeys indicates that the contribution of the protein fraction to antioxidant activity is minimal.

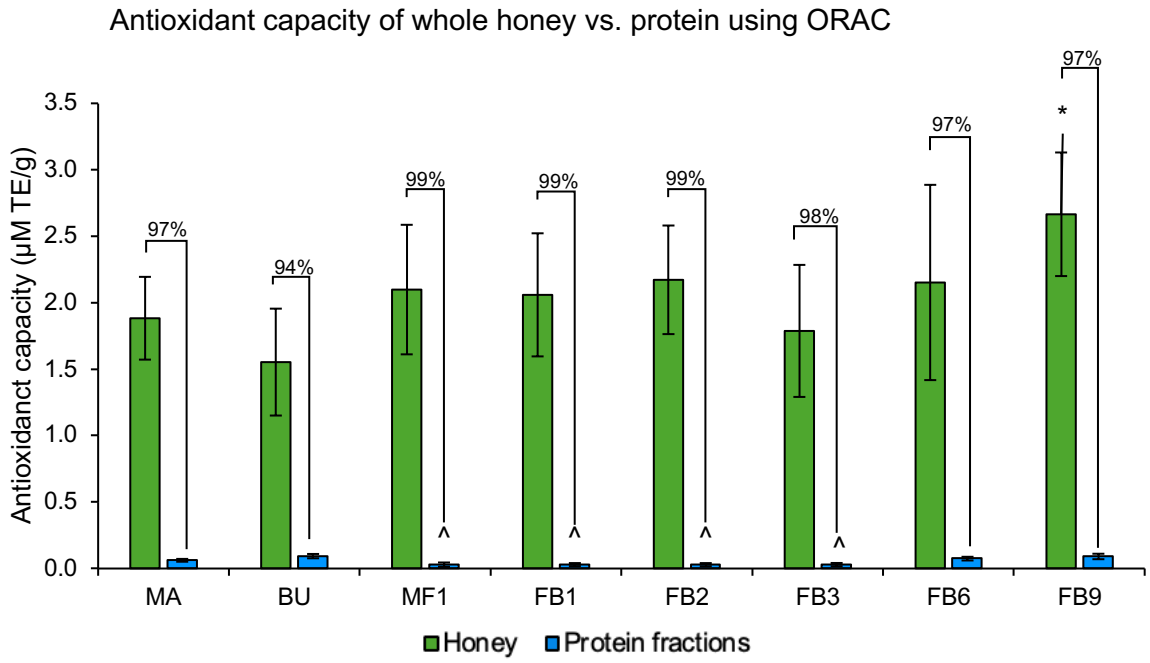


Figure 5.4.2. Antioxidant activity, ORAC assay of the honey samples and the protein fractions expressed as µM TE/g. Data is an average of three experiments ± SEM. Data is normally distributed and statistical significance was determined using ANOVA tests. Samples denoted with # are significantly different to the MA whole honey control, * are significantly different to the BU whole honey control, and ^ are significantly different to BU protein control, p≤0.05.

The ability of the honey samples and the corresponding protein fractions to induce oxidative damage was determined in the absence of AAPH (Table 5.4.1). In the HaCaT cells, the oxidative damage (refer to table 5.4.1) when investigating the CAA for the honey samples and protein samples was 95.2 ± 8.0% and 96.6 ± 5.1% for MA, 83.8 ± 4.0% and 98.7 ± 7.1% for

BU, and ranged from 82.2 ± 4.4 - 100.0 ± 8.2% and 107.9 ± 5.3 – 117.1 ± 7.3% for the FB samples, respectively. No statistical significance was observed when compared to the AAPH and MA controls. Furthermore, the induction of oxidative damage, i.e. a pro-oxidant effect, was minimal for MA, BU and the Fynbos honeys, ranging from -0.3 ± 0.1 – 0.1 ± 0.2. None of the honey samples or protein fractions at the concentration used effectively scavenged AAPH generated peroxyl radicals (Table 5.4.1).

The oxidative damage on SC-1 cells (refer to table 5.4.1) when investigating the CAA for the honey and protein samples was 93.3 ± 5.1% and 88.6 ± 5.3% for MA, 96.0 ± 3.3% and 82.2 ± 6.7% for BU, and ranged from 79.1 ± 3.1 – 95.1 ± 5.6% and 87.8 ± 4.1 – 95.0 ± 8.5% for the FB samples, respectively. No statistical significance was observed when compared to the AAPH and MA controls. Furthermore, the prooxidant activity of the honey samples was investigated, all samples showed an oxidative damage of 0.2±0.5-2.1±0.3% with no statistical significance observed. Likewise, in SC-1 cells (Table 5.4.1) a pro-oxidant effect was not observed where the % oxidative damage was minimal, 0.2 ± 0.5 – 1.6 ± 0.7% for the Fynbos honey samples which was not significantly different compared with MA. Therefore, the protein fractions did not significantly scavenge AAPH generated peroxyl radicals in both the HaCaT and SC-1 cell lines. Cellular exposure for 48 hrs does not increase the ability of the cells to effectively scavenge ROS.

Table 5.4.1. Pro-oxidant and antioxidant (% oxidative damage (%OD)) evaluated in HaCaT and SC-1 cells exposed to honey (% (v/v)) at 1.25% and 0.625%, respectively and the protein fractions analysed with the DCFH-DA assay.

		DCFH-DA/HaCaT		DCFH-DA/SC-1	
	Sample	**Pro-oxidant (% OD)***	**Antioxidant (% OD)**	**Pro-oxidant (% OD)**	**Antioxidant (% OD)**
Honey	MA	-0.1 ± 0.1	95.2 ± 8.0	2.1 ± 0.3	93.3 ± 5.1
	BU	-0.3 ± 0.1	83.8 ± 4.0	1.6 ± 0.2	96.0 ± 3.3
	FB range	-0.2 ± 0.1-0.1 ± 0.2	82.2 ± 4.4 – 100.0 ± 8.2	0.2 ± 0.5 - 1.6 ± 0.7	79.1 ± 3.1 – 95.1 ± 5.6
Protein fraction	MA	-	96.6 ± 5.1	-	88.6 ± 5.3
	BU	-	98.7 ± 7.1	-	82.2 ± 6.7
	FB range	-	107.9 ± 5.3 – 117.1 ± 7.3	-	87.8 ± 4.1 – 95.0 ± 8.5

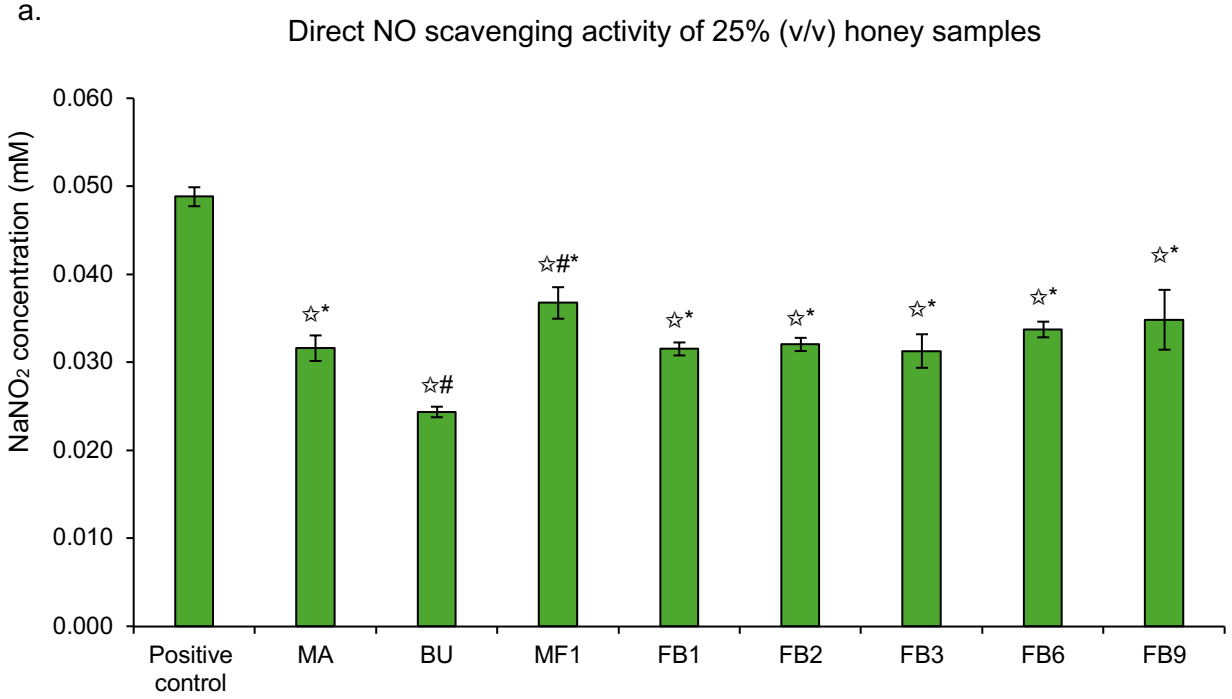
"-" is undetected
Data was expressed as oxidative damage a percentage of the AAPH positive control. Differences relative to the positive control are not significant. Differences compared to the MA control are not significant.

5.4.1 Inflammatory activity

The ability of the different honeys and the protein fractions to directly scavenge NO generated from SNP was determined (Figure 5.4.3). The NO levels following the addition of a 25% (v/v) honey (final concentration 6.25% (v/v)) was 0.03 ± 0.00 mM for MA, 0.02 ± 0.00 mM for BU, 0.04 ± 0.00 mM for MF1, and ranged from 0.03 ± 0.00 - 0.04 ± 0.00 mM for the FB samples, respectively. Differences compared with the positive control was statistically significant indicating that all honeys scavenged NO. Compared with MA, the NO scavenging was greater for BU but significantly less for MF1. Compared with BU, the NO scavenging was significantly less for all other honey samples.

For the protein fractions the NO levels were 0.014 ± 0.000 mM for MA, 0.013 ± 0.000 mM for BU, 0.014 ± 0.001 mM for MF1, and ranged from 0.013 ± 0.001 - 0.014± 0.000 mM for the FB samples, respectively. The NO levels were significantly lower than the positive control. The NO concentration for all protein fractions was similar indicating that MA and the FB protein fractions had similar NO scavenging activity. This indicates that the protein fractions do contribute to the NO scavenging activity of honey resulting in level about half that of the raw honey.

a.



b.

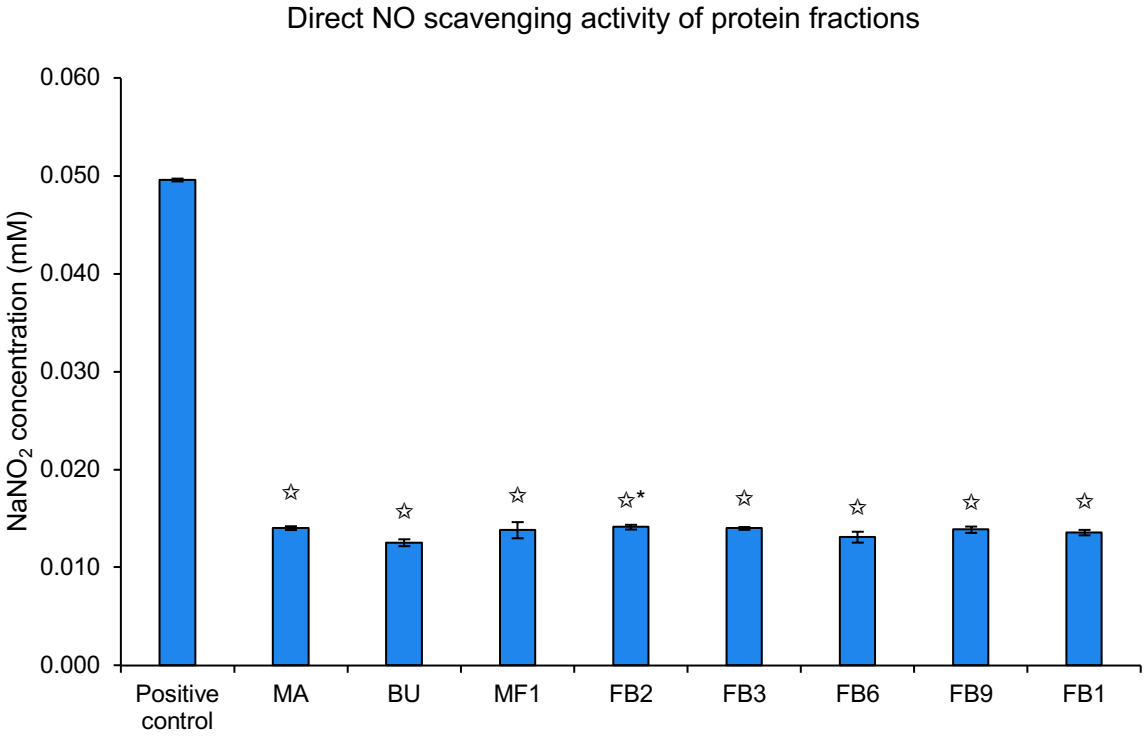


Figure 5.4.3. NO levels following the addition of (a) 25% (v/v) honey and the (b) protein fractions expressed as % NO produced by the positive control. Data is an average of three experiments ± SEM. Data is normally distributed and statistical significance was determined using ANOVA tests. Samples denoted with # are significantly different to the MA honey, * are significantly different to the BU whole honey, and ☆ are significantly different to the positive control, p≤0.05.

The anti-inflammatory activity related to the reduction of NO levels induced by LPS in RAW 264.7 cells was then evaluated for the honey samples and protein fractions (Figure 5.4.4a and b). LPS induced, 9.53 µM NO and this was reduced to 3.88 ± 1.07 µM for MA, 1.60 ± 0.25 µM for BU, 6.21 ± 2.32 µM for MF1, and ranged from 2.53 ± 0.61 - 8.78 ± 2.90 µM for the FB honeys. The BU honey had the highest anti-inflammatory activity compared with all honey samples. The NO scavenging activity of FB9 (8.78 ± 2.90 µM) was significantly lower than the MA (p=0.0353) and BU (p=0.0002) controls, with FB1, FB2, FB3 and FB6 having activity similar to MA. Samples MA (p=0.0042), BU (p=<0.0001), FB1 (4.96 ± 2.11 µM, p=0.0389), FB3 (4.54 ± 2.35 µM, p=0.0174), and FB6 (2.56 ± 0.61, p=0.002) has significantly lower NO equivalents compared to the LPS control.

The anti-inflammatory activity related to NO scavenging of the protein fractions was 17.69 ± 1.01 µM for MA, 10.45 ± 0.47 µM for BU, 10.02 ± 0.52 µM for MF1, and ranged from 10.49 ± 0.49 - 14.20 ± 0.76 µM for the FB protein fractions. The proteins lacked NO scavenging activity as NO levels were greater than the LPS control, 9.53 µM, which indicates a possible pro-inflammatory effect. Sample MA (p=0.0017) was significantly higher than the LPS control. This indicates a increased anti-inflammatory activity compared to the other protein samples, especially samples BU (p=0.0265), MF1 (p=0.0145), and FB9 (10.49 ± 0.49 µM, p=0.0282) which had significantly reduced NO equivalents compared to the MA control.

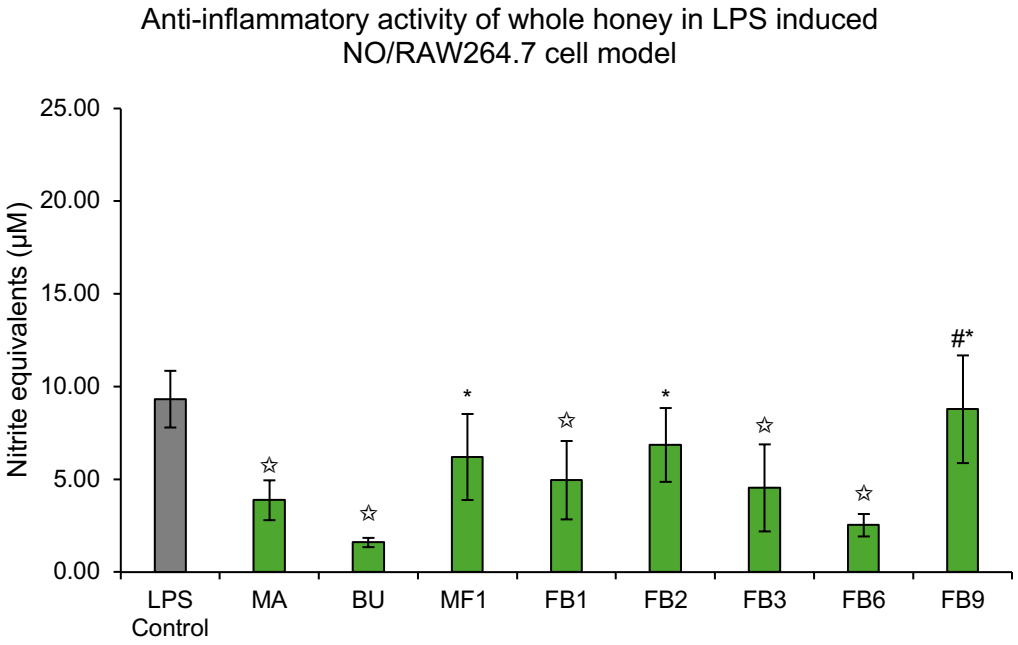
The RAW 264.7 murine macrophages were exposed to the different honeys and protein fractions, with no LPS added (Figure 5.4.5a and b). For the honey samples the induced NO was 1.72 ± 0.86 µM for MA, -0.44 ± 0.13 µM for BU, 2.24 ± 0.35 µM for MF1, and ranged from 0.72 ± 0.66 - 6.47 ± 0.95 µM for the FB samples. Samples MA (p=<0.0001), BU (p=<0.0001), MF1 (p=<0.0001), FB1 (0.92 ± 0.06 µM, p=<0.0001), FB3 (0.72 ± 0.66 µM, p=<0.0001), and FB6 (0.84 ± 0.65 µM, p=<0.0001) has significantly lower NO equivalents compared to the LPS control. These low values compared with the data generated in Figure 5.4.4.a, indicate that the honeys do have anti-inflammatory activity.

All the protein fractions induced NO formation, in the RAW264.7 cell (Figure 5.4.5b) that was 16.22 ± 1.09 µM for MA, 0.70 ± 0.14 µM for BU, 9.20 ± 1.88 µM for MF1, and ranged from 8.06 ± 1.74 - 20.88 ± 0.53 µM for the FB samples. Sample FB6 (20.88 ± 0.53 µM, p=0.0002) and FB9(18.04 ± 1.71 µM, p=0.0162) were significantly higher than the positive control (8.71 µM). Interestingly the ability of the protein fraction of BU to induce NO was significantly lower than MA (p=<0.001) and positive control (p=0.0077). The pro-inflammatory effect of the protein fractions was similar to MA, albeit slightly higher for FB6 and FB9.

With the completion of the triplicate experiments the percentage cell viability of each triplicate in both cellular models was evaluated with the CV assay (Table 5.4.2.). The percentage cell density for the anti-inflammatory (+LPS)/RAW 264.7 cell model following exposure to the honey samples was 93 ± 2% for the LPS control, 92 ± 5% for MA, 93 ± 5% for BU, 97 ± 4% for MF1, and ranged from 91 ± 5 - 99 ± 8% for the FB honey samples. For the corresponding protein fractions this was 79 ± 0 for the LPS control, 80 ± 0% for MA, 82 ± 1% for BU, 81 ± 2% for MF1, and ranged from 78 ± 4 – 83 ± 2% for the FB protein fractions. No statistically significant differences in percentage cell viability were observed compared with the untreated and MA controls

The cell density following exposure to the honey samples for the pro-inflammatory (-LPS)/RAW 264.7 cell model was 85 ± 7% for the LPS control, 92 ± 5% for MA, 92 ± 2% for BU, 97 ± 4% for MF1, and ranged from 85 ± 4 - 97 ± 4% for the FB honey samples. For the protein fractions this was 81 ± 0% for the LPS control, 77 ± 3% for MA, 93 ± 3% for BU, 88 ± 5% for MF1, and ranged from 88 ± 4 - 92 ± 3% for the FB protein fractions. No statistically significant differences in percentage cell viability were observed compared with the untreated and MA controls.

a.



b.

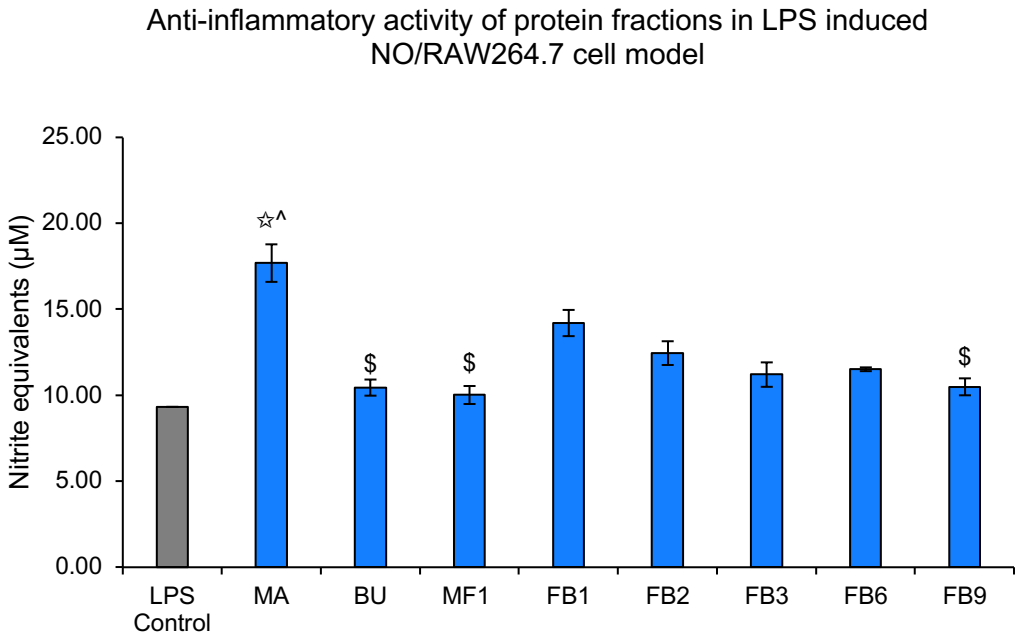
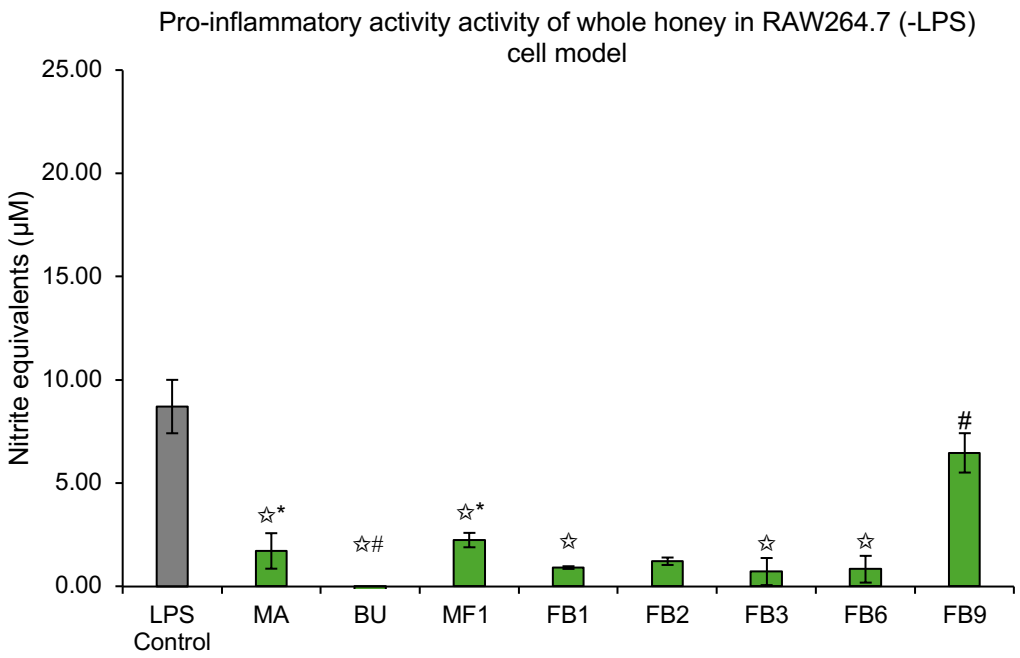


Figure 5.4.4. The anti-inflammatory (NO scavenging (+LPS)) in the RAW264.7 cells exposed to (a) 1.25% (v/v) honey and (b) protein fraction. Data was expressed as nitrite equivalents (µM). Data is an average of three experiments ± SEM. Data is normally distributed and statistical significance was determined using ANOVA tests. Samples denoted with # are significantly different to the MA honey control, * are significantly different to the BU honey control, $ are significantly different to the MA protein control, ^ are significantly different to BU protein control, and ☆ are significantly different to the +LPS control, p≤0.05.

a.



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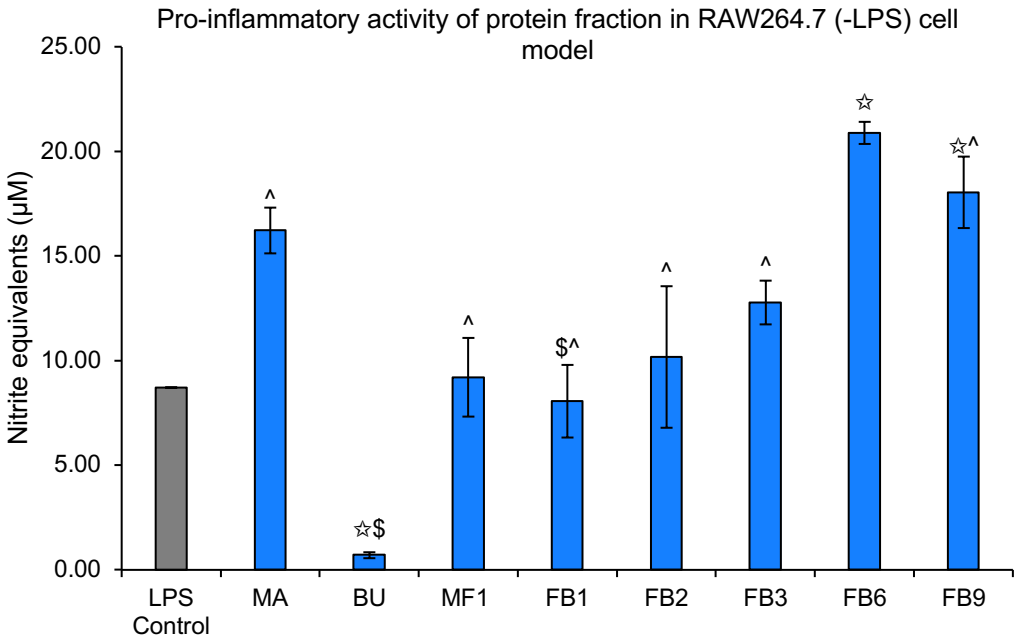

Figure 5.4.5. The pro-inflammatory (NO scavenging (-LPS)) in the RAW264.7 cells exposed to (a) 1.25% (v/v) honey and (b) protein fraction. Data was expressed as nitrite equivalents (µM). Data is an average of three experiments ± SEM. Data is normally distributed and statistical significance was determined using ANOVA tests. Samples denoted with # are significantly different to the MA honey control, * are significantly different to the BU honey control, $ are significantly different to the MA protein control, ^ are significantly different to BU protein control, and ☆ are significantly different to the +LPS control, p≤0.05.

Table 5.4.2. The percentage density of the RAW 264.7 cells after the anti- or pro-inflammatory studies evaluated with the Crystal Violet assay.

		Cell density (%)								
Sample type	**Activity**	**LPS**	**MA**	**BU**	**MF1**	**FB1**	**FB2**	**FB3**	**FB6**	**FB9**
Honey	Anti-inflammatory	93 ± 2	92 ± 5	93 ± 5	97 ± 4	95 ± 5	99 ± 8	98 ± 7	91 ± 5	99 ± 6
	Pro-inflammatory	85 ± 7	92 ± 5	92 ± 2	87 ± 6	91 ± 4	97 ± 4	85 ± 4	86 ± 4	92 ± 6
Protein	Anti-inflammatory	79 + 0	80 ± 0	82 ± 1	87 ± 2	83± ± 2	80 ± 1	80 ± 1	78 ± 4	82 ± 1
	Pro-inflammatory	81 ± 0	77 ± 3	93 ± 3	88 ± 5	90 ± 3	88 ± 4	92 ± 3	88 ± 4	91 ± 3
No statistical significance was determined										

The TPC was correlated with the antioxidant assays (ORAC, TEAC, and DCFH-DA cell models) and the anti-inflammatory assays (SNP and LPS induced NO/RAW 264.7 cell model) analysed using the Pearsons correlation test and significance was detected using ANOVA on Microsoft Excel. Significantly strong correlations were determined between the TPC and the TEAC assay with a Pearson coefficient value of 0.9586. Thereby indicating a positive linear relationship between the TPC and this antioxidant assay. Weak to no correlation was determined between the TPC and ORAC and *in vitro* DCFH-DA (both cell lines) activity with Pearson coefficient values of -0.2629 (ORAC), -0.2598 (CAA/HaCaT) and 0.4217 **(**CAA/SC-1). In addition, moderate to weak correlations were observed between the TPC and direct NO scavenging activity and the *in vitro* anti-inflammatory activity for the honey samples with Pearson coefficient values of -0.4855, and -0.6112, respectively (no significance determined). In contrast, a weak to no correlation was observed between TPC and the *in vitro* pro-inflammatory activity for the honey samples with a Pearson coefficient value of -0.1784 (no significance determined). Weak correlations indicate that other molecules and not necessary the polyphenols contribute to activity.

Table 5.4.3. Pearson coefficient (r), correlation coefficient (r^2), p values and significance* of honey and the protein fractions for TPC, the antioxidant and the anti-inflammatory assays.

	Sample	TEAC	ORAC	CAA/HaCaT cell model	CAA/SC-1 cell model
TPC	Honey	r=0.9586 r^2=0.9189 p=0.0002 **Significant*****	r=-0.2629 r^2=0.0691 p=0.0035 ns	r=-0.2598 r^2=0.0675 p=0.5344 ns	r=0.4217 r^2=0.1778 p=0.2981 ns
	Protein	r=0.9553 r^2=0.9126 p=0.0002 **Significant*****	r=0.8845 r^2=0.7824 p=0.0035 **Significant****	r=-0.4209 r^2=0.1772 p=0.2990 ns	r=-0.3918 r^2=0.1535 p=0.3370 ns
		Direct NO scavenging	**RAW 264.7 +LPS**	**RAW 264.7 – LPS**	
TPC	Honey	r=-0.4855 r^2=0.2357 p=0.2225 ns	r=-0.6112 r^2=0.3736 p=0.1074 ns	r=-0.1784 r^2=0.0318 p=0.6726 ns	
	Protein	r=-0.4761 r^2=0.2266 p=0.2331 ns	r=-0.1247 r^2=0.0155 p=0.7686 ns	r=-0.1106 r^2=0.0122 p=0.7943 ns	

All data is normally distributed.
Correlation coefficients describe the strength and direction of association between two variables
Pearson correlation is a measurement of linear association between two normally distributed variables
Statistical significance using the Pearson coefficient is indicated as P<0.05*, P<0.01**, P<0.001***, P<0.0001****

5.5 Discussion

Antioxidant and anti-inflammatory activities are significant in regulating oxidative stress and inflammation allowing for a dual immunomodulatory role.[275] Dual immunomodulation refers to the process of regulating the immune response through pharmacological intervention, genomic editing, regenerative medical tools, and natural products such as honey. Dual modulation can involve both activating and supressing the immune system ultimately regulating immune components to restore homeostasis, alleviate disease symptoms, and improve clinical outcomes by selectively attenuating destructive immune responses whilst enhancing protective antimicrobial defences.[276] For instance, transgenic models in Alzheimer's disease have shown that manipulation of immune responses provide insight into the disease pathogenesis, drawing focus to the need for careful modulation to avoid exacerbating symptoms.[284] In cancer therapies, dual modulation involves the combination of cytostatic therapies and immunomodulatory agents to strengthen immune recognition of tumours.[285] Studies have shown that combination immunomodulatory therapies can induce lead to long-term remission in highly aggressive malignancies, such as triple negative breast cancer and neuroblastoma, by reshaping the tumour microenvironment.[285] Dual immunomodulation in wound healing is significant to the process of simultaneously establishing a pro-inflammatory microenvironment to prevent bacterial infection whilst promoting an anti-inflammatory environment crucial for tissue repair.[277,278] Therefore, analysing the antioxidant and anti-inflammatory activities in wound healing research is essential for developing effective therapeutic interventions that can enhance the healing process and mitigate complications associated with oxidative stress and inflammation.

The antioxidant capacity of the Fynbos honey samples analysed using TEAC ranged between 2.05 ± 0.35 - 6.27± 0.46 µM TE/g, comparative analysis using the same TEAC parameter revealed that this antioxidant activity is either lower than or comparable to that of other South African Fynbos and Manuka honeys identified by Magoshi *et. al.*[199] [199]Furthermore, Anand *et. al.*[279] identified the antioxidant capacity of a Manuka (UMF 22+) honey and super Manuka honey (MGO-400) with values of 30.72 ± 0.27 µM TE/g and 21.28 ± 0.14 µM TE/g, respectively evaluated with the same TEAC assay parameters. Likewise, Yusof *et. al.*[280] reported the antioxidant capacity, of three different Manuka honeys with UMF10+, 15+, and 18+ as 17.22, 17.53, and 19.00 µM TE/g,[280] higher than that observed for the Fynbos samples and that of the Manuka sample (4.62 ± 0.16) evaluated in this study. However, a study conducted by Serem and Bester[152] identified the antioxidant capacity, using the TEAC assay, of various Fynbos honeys of which two samples had activity of 5.36 ± 0.5, 6.77 ± 1.3[152] comparable to

the antioxidant capacity identified in this study for samples FB1, FB6, FB9 although lower than that for BU honey.

The antioxidant capacity of the Fynbos honey samples analysed using ORAC ranged between 1.79 ± 0.41 - 2.67 ± 0.73 µM TE/g, and when compared to that of other Fynbos honeys, the antioxidant capacity was lower, where a concentration range of 20.74 ± 2.78 - 63.66 ± 8.22 µM TE/g was reported for 6 different Fynbos samples by Magoshi *et. al.*[199] using the same ORAC parameter.[199] For MA the value was 1.88 ± 0.31 µM TE/g lower than 48.41 ± 14.1 µM TE/g for the Manuka honey identified by Magoshi *et. al.*,[199] 11.60 ± 0.027 µM TE/g for Buckwheat honey identified by Baretta *et. al.*,281 and 24.82 ± 0.5 µM TE/g and 12.40 ± 0.3 µM TE/g for a Manuka (UMF 22+) honey and super Manuka honey (MGO-400) identified by Anand *et. al*.[279] The variations between the MA sample used in this study and in Magoshi *et. al.* could be due to differences in environmental conditions resulting in variation of the honey components.[156]

Polyphenols, including those found in honey, have been observed to scavenge free radicals through two distinct mechanisms known as hydrogen atom transfer and single electron transfer. In hydrogen atom transfer, a hydrogen atom is donated from the OH group within the polyphenol structure and transferred to the free radical neutralising its high reactivity.[282] The OH group of the polyphenol is considered a highly reactive site due to the relatively low bond dissociation enthalpy.[292,293] After donating the hydrogen atom the polyphenol becomes a phenoxyl radical that is generally more stable because of the delocalisation of unpaired electrons across the aromatic ring.[292,293] The phenoxyl radical further reacts with other free radicals or is neutralized by other antioxidant molecules in the system.[292,293] The electrons within the hydroxyl structure have a conjugation effect weakening the binding strength of the H-atom subsequently allowing for easy disassociation to neutralise free radicals.[283] In single electron transfer, the hydroxyl group of the polyphenol molecule readily donates a single electron to unstable free radical like superoxide anions hydroxyl radicals ultimately preventing DNA, lipid and protein oxidative damage.[291,295] Upon donating an electron, the polyphenol becomes a radical cation, which is more stable than the ROS due to the resonance delocalization of the cation, distributing the unpaired electron across the benzene ring, further stabilizing the structure.[291,295] This resonance stabilization helps maintain the structural integrity of the polyphenol radical cation, with its stability largely influenced by the number and arrangement of hydroxyl groups. [291,295]

Furthermore, polyphenols exhibit metal chelating antioxidant activity by binding to transition metals like iron and copper.[296] The hydroxyl group in the phenolic structure act as binding sites for this transition metal ions subsequently forming a stable complex neutralising the pro-oxidant activity of the metal ions. When unbound, iron and copper readily participate in redox reactions producing highly reactive ROS. For example, by chelating iron the polyphenols inhibit the Fenton reaction subsequently inhibiting the production of OH radicals.

The lack of antioxidant activity observed for the protein fractions is potentially related to the low polyphenol content (Figure 3.4.4). Additionally, one can hypothesise that the lower concentration rates could be due to differences in geographical location of the various honeys.[297] Additionally, variation observed between the Fynbos honey data identified in this study and by Magoshi *et. al*.[199] could be due to different beekeeping practices of the various samples, for examples, different harvesting times between different South African beekeeping farms.[297]

To further evaluate the relevance of these findings, the protective capacity of the honey and protein samples against AAPH-generated peroxyl radical damage was analysed after 48 hours in HaCaT and SC-1 cells using the DCFH-DA assay. This assay is analogous to the ORAC assay in an in vitro setting, where AAPH is used to generate peroxyl radicals.[154] The DCFH-DA/HaCaT cell model and DCFH-DA/SC-1 cell model provides a more physiologically relevant environment as keratinocyte and fibroblast cells are key role players in tissue regeneration and repair discussed in Chapter 2. Keratinocytes are mainly responsible for re-epithelialisation, skin homeostasis, and pathophysiology of scar formation. In addition, keratinocytes, have several ROS protection mechanisms and these are enzymatic involving superoxide dismutase (SOD), catalase, and glutathione peroxidase and antioxidant molecules such as glutathione (GSH).[284] Fibroblasts are responsible for new ECM formation to support granulation tissue and increase in collagen synthesis.[214] Fibroblasts also exhibit ROS protection due to the presence of antioxidant enzymes,[285] and thioredoxin/thioredoxin reductase, that reduces oxidized proteins.[286] Other fibroblasts cell models such as primary dermal human fibroblasts (PDHF cell) would be a more representative model of the human epidermal tissue target, however complex ethical considerations surrounding this model would be needed. Human cells offer a direct translation to clinical applications as they better reflect the complex biology of the human integument.[301]

AAPH radicals induce 100% oxidative damage in the HaCaT and SC-1 cells. Relative to the positive control (100% OD), no CAA was observed for the control honeys and Fynbos honeys

at a final concentration of 1.25% (v/v) in HaCaT cells and 0.625% (v/v) in SC-1 cells. This lack of CAA is potentially related to the concentrations used and/or the experimental design.

A study done by Gohar *et. al.*[287] isolated the protein from monofloral Ziziphus species honey in Himalayan and Pothohar plateau regions in northern Pakistan, using gel filtration chromatography where the precipitation proteins was performed with a AKTA FPLC system and Hiload 16/60 Superdex 200 prep grade column. Using the ROS inhibitory/polymorphonuclear leukocyte cell model, isolated polymorphonuclear leukocytes were activated by 250 µg/mL of serum opsonized zymosan-A. The precipitated crude honey proteins were added in varying concentration at 400, 40, 4, and 0.4 ng/mL, and incubated for 20 min at room temperature. In this study by Gohar *et. al.*[287] the ROS formation was inhibited by the varying honey proteins. Differences between this and the present study is potentially related to the cell lines used and their response to radicals where leukocytes as part of the innate immune system have biochemical pathways that exhibit a robust mechanism of scavenging O^{2-} and responding to inflammatory stimuli.[288] The innate immune system uses different ROS scavenging strategies in leukocytes compared to fibroblasts and keratinocytes. In leukocytes, particularly phagocytes, ROS production occurs through a respiratory burst driven by the NADPH oxidase complex, generating O^{2-} and H_2O_2 to eliminate pathogens.[304–306] However, this process is tightly controlled to prevent excessive ROS accumulation, which can lead to cellular damage and apoptosis.[304–306] In contrast, fibroblasts and keratinocytes primarily rely on enzymatic antioxidant defences, such as superoxide dismutase and catalase, to counteract oxidative stress and preserve cellular integrity during wound healing and tissue repair.[304–306]

In the study of Serem and Bester[152] the CAA (described as cellular protective effect) was determined using the DCFH-DA assay at 0.025% and 2.5% (v/v) of whole honey (final concentrations) of several South African Fynbos honey samples. In the study of Serem and Bester[152] cells were exposed to AAPH radical for 1 h whereas in this study the AAPH was exposed for 48 h and treated with 1.25% (v/) of South African Fynbos honey samples. Although the samples in this study and those discussed for Serem and Bester[152] are of similar geographical origin, the time at which they were harvested could be different, environmental factors such as drastic weather changes during different seasons may account for the differences in CAA activity observed. For example, the abundance of nectar during peak floral periods in spring and summer often results in higher honey production and potentially better quality.[307] Furthermore, prolonged exposure to AAPH, such as the 48 h exposure time used in this study, leads to an imbalance of ROS production and cellular antioxidant defence.[308]

AAPH decomposes to form carbon-centred radicals, initiating lipid peroxidation and increasing ROS production in the presence of oxygen and polyunsaturated fatty acids.[309] A study conducted by Barygina *et. al.*[310] exposed human keratinocytes (HaCaT) to 0.5 M AAPH for 24 hours, resulting in sustained increase in NADPH oxidase activity up to 48 h following AAPH removal, elevating levels of H_2O_2 in the extracellular medium causing cellular damage.[310] Additionally, prolonged exposure to AAPH (50 mM for 2 hours) decreased cell viability in HepG2 cells from 96.9% to 68.4%, indicating that increased ROS levels correlate with cellular damage.[309] Therefore in this prolonged exposure to AAPH causes cumulative increase in ROS concentration overwhelming the cellular antioxidant defence response, potentially increasing cellular damage and demonstrating higher rates of cytotoxicity.[289]

The antioxidant activity of samples BU, FB6, and FB9, the darker honeys, consistently had the highest antioxidant activity across the ORAC, TEAC, and DCFH-DA assays, which is to be expected as literature has reported strong correlations between honey colour and antioxidant activity.[290,291] In the present study (Table 5.4.3) a strong correlation was observed between the TPC vs TEAC ($r=0.9586$, $r^2=0.9189$, $p=0.0002$). Weak correlations between TPC vs ORAC ($r=-0.2629$, $r^2=0.0691$, $p=0.0035$), TPC vs DCFH-DA/HaCaT cell model ($r=-0.2598$, $r^2=0.0675$, $p=0.5344$), and TPC vs DCFH-DA/SC-1 cell model ($r=0.4217$, $r^2=0.1778$, $p=0.2981$) were determined. This may highlight the complexity of factors and the types of molecules that contribute to the antioxidant activity of honey.

The correlation between TEAC and TPC tends to be stronger than that of ORAC and DCFH-DA assay with TPC due to differences in their chemical mechanisms. TEAC, which measures the ability of antioxidants to scavenge $ABTS^+$ radicals via a single electron transfer aligning with the F–C reagent used to determine TPC, which also operates via single electron transfer.[314] Since many polyphenols act as single electron transfer-based antioxidants, their contribution to TEAC values is largely proportional to their presence, leading to a strong correlation.[315] In contrast, ORAC primarily operates via a hydrogen atom transfer mechanism, which is more influenced by the structure and polymerization of polyphenols, as well as the presence of non-phenolic hydrogen donors, thereby reducing correlation strength.[268] ORAC's reaction kinetics are also time-dependent, further contributing to variability in its relationship with TPC.[316] Weak correlation is further observed with DCFH-DA, which measures intracellular ROS scavenging rather than direct antioxidant capacity, as it is affected by polyphenol bioavailability, metabolic transformations such as glucuronidation, and indirect antioxidant effects via pathways like Nrf2 activation.[317]

The antioxidant activity evaluated in this study was below the values reported in literature and this may be related differences between the geographical origins of the samples, poor processing and storage conditions during harvesting, and prolonged sample dilution.[292] Poor processing procedures include heat and light exposure that leads to the degradation of the sensitive phytochemicals. Kačániová *et. al.*[255] identified that honey samples diluted to 50%, 25%, 12.5%, and 6.25% elucidated a significant dilution related decrease in antioxidant capacity.[292] In summary, low antioxidant values in these assays are often a result of both intrinsic factors related to the antioxidants themselves and extrinsic factors such as environmental conditions, processing/storage choices, and methodical conditions.

Due to limited research, undertaken on the protein fractions of honey, no studies were found evaluating the antioxidant activity of this fraction using the TEAC and ORAC assays. Nonetheless, activity determined in the present study is minimal. The antioxidant capacity of the Fynbos protein fractions analysed using TEAC ranged between 0.13 ± 0.05 - 0.22 ± 0.06 µM TE/g and was lower compared with their honey counterparts due to the lack of polyphenols in these samples (Figure 3.4.4). The antioxidant capacity of the Fynbos protein fractions, measured using the ORAC assay, ranged from 0.03 ± 0.01 - 0.09 ± 0.01 µM TE/g (Figure 5.4.2). This activity was lower in comparison to their honey counterparts where the presence of polyphenols contributes to activity (Figure 3.4.4). Although polyphenols are key contributors, proteins and peptides also exhibit antioxidant activity through metal chelation and radical scavenging.[319] However, the pooling of fractions leads to a decrease in protein concentration due to overlap with high sugar content, potentially resulting in the loss of peptides and proteins that may contribute to antioxidant activity. Without specific identification of protein profiles in whole honey and pooled protein fractions using LC-MS/MS, any discussion of their precise mechanisms of action would remain speculative.

Moreover, studies reporting the correlations between honey protein and colour, antioxidant content and capacity have not been found. For these samples (Table 5.4.3) strong correlations were found between the TPC vs ORAC ($r=0.8845$, $r^2=0.7824$, $p=0.003$) and TPC vs TEAC ($r=0.9553$, $r^2=0.9126$, $p=0.0002$), indicating a positive correlation between TPC and the different antioxidant assays. A weak correlation between TPC vs DCFH-DA/HaCaT and DCFH-DA/SC-1 were observed, reflecting the complexity of honey where ascorbic acid, amino acids and peptides can also contribute to antioxidant activity.[290,293] Despite this, it is important to note that although low statistical correlation between two variables is indicative of nonlinear relationship; this does not mean that a relationship between the two variables is absent, it simply means the relationship is not linear. As mentioned previously, the different chemical

mechanisms of action do not align linearly with the chemical mechanism of TPC, given that TEAC relies on direct electron donation, while ORAC is structure- and kinetics-dependent, and DCFH-DA is influenced by cellular metabolism.[314–317]

The antioxidant capacity of the Fynbos protein fractions was lower than the corresponding honey, due to the low TPC of these samples (Figure 3.4.4). As the correlations for TPC vs ORAC and TPC vs TEAC are strong positive correlations, confirming the directly proportional linear relationship between the two factors, a low TPC content is associated with low antioxidant capacity identified with the ORAC or TEAC assays.

The NO scavenging assay reduced NO levels from 100% to 62 ± 2.6 – 68 ± 1.5% for the Fynbos honey. The NO scavenging ability of Fynbos honey reported by Magoshi *et. al.*[199] ranged between 36 - 60%.[199] The higher % NO scavenging of the Fynbos honey identified by Magoshi *et. al.*[199] may be related to differences in the concentrations used, as Magoshi *et. al.*[199] used 10% (v/v) Fynbos honey, therefore a lower NO scavenging ability observed in this study could be expected as honey samples from the same region were used. It has been observed that the anti-inflammatory activity of honey does not increase in a perfectly linear manner with increasing concentration; multiple studies have observed a more complex relationship, primarily indicating a plateau effect where further concentration increase does not significantly enhance the anti-inflammatory effect.[282] Specifically a study done by Minden-Birkenmaier *et. al.*[321] identified 0.5% Manuka honeys ability to improve the release of CXCL8/IL-8, CCL2/MCP-1, CCL4/MIP-1β, CCL20/MIP-3α, IL-4, IL-1ra, and FGF-13 while reducing proteinase 3 release in dHL-60 cellular model. Whereas the higher dose, 3% Manuka honey, significantly increased the release of TNF-α and CXCL8/IL-8 simultaneously decreasing the release of all other analytes.[282,321] In addition, the ability of the honey samples to induce NO was also investigated. The range of NO concentrations induced by 1.25% Fynbos honey was 0.72 ± 0.66 - 6.47 ± 0.95 µM.

In a cellular model, the anti- and pro-inflammatory activity related to NO scavenging and induction were subsequently assessed using the +LPS induced NO/RAW 264.7 cell model or -LPS induced NO/RAW 264.7 cell model respectively. The 1.25% (v/v) Fynbos honey reduced NO levels from 9.32 ± 1.53 µM to 2.53 ± 0.61 - 8.78 ± 2.90 µM (Figure 5.4.4a) indicating an anti-inflammatory effect. The NO scavenging ability of medical grade lozenge and drop formulations (20 – 100 µg/mL) of multifloral Canadian honey after 24 h exposure in the +LPS/IFN-γ induced NO/RAW 264.7 murine macrophage model reduced NO levels from 24.30 µM to 19.96 µM and 13.02 µM. [294] The NO concentration determined by Larsen *et. al.*[294]

for the medical grade Canadian honey lozenge and drop formulations was higher compared to that produced in this current study which utilised the same *in vitro* model. Thereby suggesting an improved anti-inflammatory potential of the Fynbos honey samples used in this research compared to the medical grade Canadian honey lozenge and drop formulations analysed by Larsen *et. al*.[294]

The enzyme iNOS produces NO from L-arginine,[295] electrons are transferred from NADPH to the haeme of the oxygenase domain of iNOS subsequently oxidizing the guanidine residue in L-arginine to produce NO and l-citruline.[295] This pathway is initiated by bacterial LPS stimulation through the NF-kB and activating protein-1 transcription factors.[296] Polyphenols, such as those found in honey have shown the ability to regulate the intracellular pathway of NO production through the downregulation of NF-kB pathways and reduction of the binding complex of NF-kB DNA in the promotor region of COX-2 and iNOS subsequently reducing the NO production.[297]

In the NO scavenging assay, the protein fractions reduced NO levels from 100% to 26 ± 0.9 - 28± 0.7%. The protein fractions effectively scavenged NO indicating that peptides and/or proteins in this fraction have direct NO scavenging properties. The MRJP family of proteins function in anti-inflammatory activity, specifically MRJP1, MRJP 2, and MRJP3. MRJP1 and MRJP2 have shown their ability to stimulate TNF-α and immunoglobulin E, whilst MRJP3 has been observed to supress pro-inflammatory cytokines in mice models.[298–301] However, the presence of these proteins must be further identified to accurately detect their link to anti-inflammatory activity. Then the anti- and pro-inflammatory activity related to NO scavenging and induction were assessed using the +LPS induced NO/RAW 264.7 cell model or -LPS induced NO/RAW 264.7 cell model respectively. The Fynbos protein fractions increased the LPS induced NO levels from 9.32 ± 1.53 µM to 10.49 ± 0.49 -14.20 ± 0.76 µM (Figure 5.4.5b). This is in contrast to the direct NO scavenging activity and may reflect the complexity of this model, where NO levels are determined by the induction of the enzyme iNOS and the subsequent release of NO.[302]

The ability of the protein fractions to induce NO was also investigated. The pro-inflammatory activity of the protein fraction in the for the FB samples was 8.06 ± 1.74 µM to 20.88 ± 0.53 µM (Figure 5.4.5b). This study identifies the pro-inflammatory activity of the protein fraction. This highlights an important aspect of antioxidant activity, where in a physiological environment NO can combine with ROS forming reactive nitrogen species, that have greater oxidative potential than peroxyl radicals. Therefore, it was observed in this study that the

protein samples contain potential pro-inflammatory proteins, that increased NO concentration, observed in a reaction with AAPH generated peroxyl radicals generates species with increased oxidative potential and this may account for the lower than expected CAA observed in Table 5.4.1.

Pro-inflammatory cytokines, such as TNF, IL-1, and IL-6, are essential in promoting the initial wound healing process by promoting inflammation, recruiting immune cells, and initiating tissue repair mechanisms.[303] The pro-inflammatory effect of honey proteins is believed to be regulated through the activation of toll-like receptors on immune cells causing the production of several pro-inflammatory cytokines, such as TNF-α and IL-1.[332] Although, pro-inflammation is often viewed negatively, it is important to take note of just how essential this activity is for chronic wounds, the dysregulation of pro-inflammatory cytokines and chemokines can prevent healthy healing.[303] The increase in pro-inflammatory activity of the protein isolates can lead to improved therapeutic techniques for chronic wounds and enhanced patient outcomes.[304]

Correlations between TPC vs NO scavenging, TPC vs +LPS/RAW 264.7 cells, and TPC vs -LPS/RAW 264.7 was 0.4458, 0.1350, and 0.1247 (Table 5.4.3), respectively. These weak correlations suggest this increase in anti-inflammatory or pro-inflammatory activity, is not attributed to the presence of polyphenols but rather peptides or proteins in the protein fractions. To further elucidate the protein component responsible for this increase in activity, liquid chromatography–mass spectrometry (LC-MS/MS) could be used to identify the protein profiles within each protein isolate. Further elucidation of these effects using enzyme-linked immunosorbent assays and reverse transcription polymerase chain reaction assays[305] to allow for quantification of specific biomarkers such as IL-1β and TNF-α providing key insight into the inflammatory response triggered by the honey protein fractions is recommended for future studies.

5.6 Conclusion

Based on the results obtained from the ORAC, TEAC, and DCFH-DA assays, the antioxidant capacity of the Fynbos honey samples, measured using ORAC and TEAC assays, showed moderate activity compared to other Fynbos and Manuka (UMF 22+ and UMF 150+) honeys identified by Baretta *et. al.,*[281] Anand *et. al.*,[279] Yusof *et. al.*,[280] and interobserver studies conducted by Serem and Bester[152] and Magoshi *et. al*.[199] The oxidative damage on HaCaT cells determined using the DCFH-DA assay for all samples was >80%, indicating a poor cellular antioxidant capacity. Interestingly, all Fynbos protein fractions and the MF1 protein fraction had an OD > 100% possibly indicating a pro-oxidant effect of the protein isolates.

Moreover, the cellular antioxidant capacity seems to be improved in the SC-1 cells whose %OD was less than that identified for the HaCaT cells. Furthermore, the direct NO scavenging by the protein fractions is greater than the honey samples but was not observed using an *in vitro* model of anti-inflammatory activity and in contrast a pro-inflammatory effect was observed.

In conclusion, this research highlights the complexity of antioxidant and inflammatory mechanisms of honey where further characterisation of the proteins involved, and pathway elucidation is required.

Chapter 6: The antibacterial activity of selected South African honeys and protein extracts

6.1 Introduction

Antimicrobial resistance (AMR) which is the resistance of microbes to approved and used therapies, is becoming increasingly prevalent in the health industry. An even more worrying form of antimicrobial resistance is multidrug-resistant (MDR) organisms,[89,306] these organisms have acquired an insensitivity or resistance to at least of one antimicrobial agent in more than three antimicrobial classes, consequently decreasing the efficacy of these antimicrobial agents against the organism.[307–309] If this issue remains unaddressed, it is projected that AMR could result in up to 50 million deaths worldwide annually by 2050.[89] The characteristics of these pathogens reduce their susceptibility to known treatments, and with the scarcity of new antibiotics, there is an urgent need for novel treatments to combat MDR infections.[89]

In this study, the antibacterial activity of various honey types was evaluated against the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *E. coli*. *S. aureus* is a Gram-positive cocci found in clusters, this type of bacteria is known to causes inflammatory diseases, including skin infections, septic arthritis, and abscesses.[310] Wounds infected with *S. aureus* are at high risk of developing *S. aureus* –related bacteraemia and associated with AMR related mortality.[311] *E. coli* is a Gram-negative bacillus being one of the leading causes of wound infection at risk of *E. coli*-bacteraemia and AMR related infections.[312] Bacteraemia is a unique trait of metastatic infection in majority of the organ systems in the body.[313]

Antibacterial activity is due to the presence of various constituents such as MGO in Manuka honey, H_2O_2 in Buckwheat honey, phenolic compounds, high sugar content, low pH, DF-1, present at various levels, suggesting a multifaceted synergistic mechanism of action.

Exposure to Manuka honey regulates bacterial size and shape consequently affecting the septal ring involved in cell division of *B. subtilis*168, *S. aureus* ATCC 25923, *E. coli* O157:H7, and *P. aeruginosa* PAO1 (ATCC 15692).[314] Tonks[315] reported that following exposure to Manuka honey (4%, w/v) smaller *Bacillus subtilis* (*B. subtilis*) and *S. aureus* bacteria cells contained condensed chromosomes indicating inhibition of cell division.[315] Furthermore, Kumar *et. al.*[316] reported differences in the response of bacteria to Manuka honey with antibacterial activity against Gram-positive *Enterococcus faecalis*, but not Gram-negative *E. coli*.[316] Matzen *et. al.*[317] also reported that honey from several different botanical origins, had antibacterial activity against Gram-positive *S. aureus* and *Staphylococcus epidermidis* but not Gram-negative *E. coli* and *P. aeruginosa.*[317]

In contrast studies conducted by Escuredo *et. al*.,[318] Isla *et. al.*,[319] Fyfe *et. al.*,[320] and Mohapatra *et. al.*[321] determined an increased susceptibility of Gram-negative compared with in Gram-positive bacterial strains.[318–321] Mohapatra *et. al.*[321] identified that the increased susceptibility of Gram-negative bacteria was due to the higher H_2O_2 content and osmolarity of the honey samples.[321] Rabie *et. al.*[322] reported that MGO effectively inhibited Gram-positive, *B. subtilis* and *S. aureus at an* MIC of 0.8 mM and 1.2 mM respectively. MGO inhibited Gram-negative, *P. aeruginosa* and *E. coli* at a MIC of 1.0 mM and 1.2 mM respectively. This indicates that MGO in honey inhibits the growth of bacteria, and inhibition is dependent on the MGO concentration in a honey type, where medical grade Manuka honey with high MGO content effectively inhibits bacterial growth.[322]

Information on the antibacterial activity of FB honey is limited to the study by Basson and Gobler.[153] testing the minimum inhibitory concentration (MIC) of FB honey against several *Streptococcus* spp, *S. aureus* (NCTC 8530), *E. coli* (NTCT 9001), and *Candida albicans* (*C. albicans* NCPF 3118). This study highlighted the efficacy of South African FB honey against various bacterial strains focussing attention on the antimicrobial activity of South African honey as a potential alternative therapy against the AMR crisis.

The aim of the research undertaken in this chapter is to determine the MIC of FB honey and the protein fractions against a model Gram-negative and -positive bacteria, *S. aureus* (DSM 2569) and *E. coli* (ATCC 700928) respectively.

6.2 Materials

Honey samples

The same honey samples and protein fractions used in previous chapters further evaluated in this chapter.

Reagents, equipment and disposable plasticware

In addition to the reagents used previously, Mueller Hinton (M-H) agar and broth (Sigma Aldrich, Atlasville, SA) was used.

Additional equipment used was a Nuaire incubator, Epoch (Biotek Instruments, Inc., Winooski, USA), Esco Laminar Flow Cabinet (Labotech, Gauteng SA), OPTIZEN POP UV/Vis spectrophotometer (KLAB, Daejeon, Republic of Korea), microbiological safety cabinet (BIOBASE, Lasec, Gauteng, SA), and SHK-IN microbiological incubator (FMH instruments, Labotech, Gauteng g SA).

Disposable plasticware was the same and used previously and in addition inoculating loops, and Petri dishes (Lasec, Western Cape, SA) were used.

Bacteria

S. aureus (DSM 2569) and *E. coli* (ATCC 700928) sourced from the America Type cell Collection (ATCC), The Global Bioresource Centre, Virginia, USA.

6.3 Methods

Minimum inhibitory concentration (50%)

Antimicrobial susceptibility testing involves determining the minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism. The MIC represents the in vitro concentration needed to suppress the growth of various bacterial strains and is typically the first phase of screening.[324]

The microbroth dilution assay was used to determine the MIC of the honey and protein fractions, the procedure was adapted from the European Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical Microbiology and Infectious Diseases.[325] *S. aureus* (DSM 2569) and *E. coli* (ATCC 700928) colonies were grown on Mueller Hinton (M-H) agar plates following the streak plate procedure adapted from Sanders.[326] A sterile inoculating loop was used to collect bacteria from either stock. The collected bacteria colonies was spread in close parallel streaks over the first quadrant of the agar plate.[326] The plate was turned 90° and the same inoculating loop was lightly swept through the inoculated area and streaked into the next quadrant.[326] This step was repeated until all quadrants of the plate had been inoculated, taking caution to prevent overlap between quadrants.[326] The plate was incubated for 24 h at 37°C and 5% CO_2.[326] The overnight cultures were used within the 18-24 h window, 2-3 isolated colonies were collected using a sterile inoculating loop and diluted in M-H broth to 0.5 McFarland (OD_{600} = 0.1).[325] The volume needed was determined using the following calculation:

Volume needed from suspension (µL) = (0.001)(Final vol. of bacterial inoculum needed (µL)) / OD600 of bacterial suspension

A 50 µL volume of sterile 50% (v/v) honey samples and 0.044 - 0.126 mg/g protein (final concentration 0.0044 - 0.0126 mg/g) was added to 50 µL of inoculum in each well of a 96 well plate. Each sample was serially diluted (2-fold) in 7 wells.[325] A 100 µL M-H broth blank, 100

µL of inoculum growth control, and 50 µL inoculum and 50 µL vehicle control was used. All plates were incubated for 24 h at 37°C and 5% CO_2. The OD was measured at 600 nm exactly 24 h later using the Epoch spectrophotometer (Biotek Instruments, Inc., Winooski, USA). The MIC_{50} of each sample was determined using GraphPad prism software version 9.5.0 (GraphPad Software, Boston, Massachusetts USA, www.graphpad).

Data management and statistical analysis

Data analysed were an average of three experiments where each measurement was done in triplicate, subsequently, 9 data points were generated per sample (independent variable) treated as dependent variables. All data was expressed as mean ± SEM of the triplicate experiments analysed using Microsoft 365 Excel 2023. MIC_{50} was determined using non-linear regression using GraphPad prism software version 9.5.0 (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Determination of parametric and non-parametric data was conducted using the D'Agostino and Pearson test; and Shapiro-Wilk test also using the GraphPad prism software version 9.5.0. Data was statistically analysed for significant differences using one-way ANOVA and Tukey's multiple comparisons test was conducted for comparison of means using the same statistical software.

6.4 Results

The MIC_{50} against *E. coli* (ATCC 700928) for the honey was 45.7 ± 6.4 mg/mL for MA, 29.9 ± 6.4 mg/mL for BU, 26.8 ± 6.4 mg/mL for MF1, FB2 lacked activity (> 500 mg/g) and for the remaining FB honey the range was 26.1 ± 3.4 – 70.0 ± 2.2 mg/mL (Table 6.4.1), with the highest activity for FB6. For the protein fractions, the MIC_{50} was 0.36 ± 0.06 mg/mL for MA, and above the highest concentration evaluated for BU, FB2, FB3, FB6 and FB9. The MIC_{50} for MF1 and FB1 was 0.11 ± 0.04 mg/mL and 0.13 ± 0.04 mg/mL respectively (Table 6.4.1). For MF1 and FB1, the protein fraction is effective against the Gram-negative bacteria.

The MIC_{50} against *S. aureus* (DSM 2569) for the honey was 280.7 ± 68.5 mg/mL for MA, and no activity for BU and MF1. For FB honey, FB2 lacked activity while the range for the remaining FB honey samples was 4.0 ± 0.9 - 391.7 ± 155.6 mg/mL (Table 6.4.1) with the highest activity for FB9. The MIC_{50} for the protein fractions against *S. aureus* (DSM 2569) was 0.14 ± 0.08 mg/mL for MA, and no antibacterial activity detected for BU, FB3, FB6 and FB9. MF1 was the most active with a MIC_{50} of 0.01 ± 0.01 mg/mL (Table 6.4.1). For FB1 and FB2, the MIC_{50} was 0.05 ± 0.04 and 0.09 ± 0.06 mg/mL respectively, with FB1 having the best antibacterial activity.

As antibacterial activity was observed for the MA honey and protein fractions against both bacteria, statistical analysis was relative to MA.

For the FB honey samples, FB6 exhibited the highest activity against *E. coli*, while FB6 and FB9 were the most effective against *S. aureus*. Their MIC_{50} values were several-fold lower than those of MA, indicating superior antibacterial activity compared to MA. For *E. coli* the MIC_{50} of MA, FB1, FB3, FB6 and FB9 was lower than *S. aureus*. In contrast, FB9 had a lower MIC against *S. aureus* than *E. coli*. These differences indicate differences in the mode of inhibition. For the protein fractions, MA and FB1 had lower MIC_{50} values for *S. aureus* than *E. coli*.

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Table 6.4.1. The MIC_{50} of the honey and protein fractions against *E. coli* (ATCC 700928) and *S. aureus* (DSM 2569).

Bacteria	MIC_{50}	MA	BU	MF1	FB1	FB2	FB3	FB6	FB9
E. coli	Honey (% (v/v)) [mg/mL]	4.6 ± 0.6 [45.7 ± 6.4]	3.0 ± 0.6 [29.9 ± 6.4]	2.7 ± 0.6 [26.8 ± 6.4]	7.0 ± 0.2 [70 ± 2.2]	> 50 [> 500]	4.8 ± 0.9 [48 ± 9.1]	2.6 ± 0.3 [26.1 ± 3.4]	5.3 ± 0.9 [52.9 ± 9.2]
	Protein fraction (mg/mL) [mg/g]	0.36 ± 0.06 [0.24 ± 0.02]	>0.11 [> 0.15]	0.11 ± 0.04 [0.07 ± 0.02]	0.13 ± 0.04 [0.07 ± 0.01][b]	>0.05 [> 0.07]	>0.04 [> 0.04]	>0.10 [> 0.13][a]	>0.01 [> 0.12][a]
S. aureus	Honey (% (v/v)) [mg/mL]	28.1 ± 6.9 [280.7 ± 68.5]	> 50 [> 500]	> 50 [> 500]	0.7 ± 0.2 [6.9 ± 1.5]	> 50 [> 500]	39.2 ± 15.6 [391.7 ± 155.6]	31.7 ± 11.0 [317.4 ± 110.4]	0.4 ± 0.1 [4.0 ± 0.90]
	Protein fraction (mg/mL) [mg/g]	0.14 ± 0.08 [0.09 ± 0.03]	>0.11 [> 0.15]	0.01 ± 0.01 [< 0.01]	0.05 ± 0.04 [0.03 ± 0.01]	0.09 ± 0.06 [0.06 ± 0.02]	>0.04 [> 0.04]	>0.10 [> 0.13]	>0.01 [> 0.12]

One way ANOVA testing was used to compare Fynbos samples to Manuka and *Buckwheat controls. Statistical significance was determined for whole honey data points for [a]*E. coli* compared to the same sample data points for [b]*S. aureus*. Statistical significance was determined for protein data points for [d]*E. coli* compared the same sample data points for [c]*S. aureus*.
Statistical significance to MF1 protein fraction ([a]) or FB6 protein fraction ([b])
$P<0.05^{a}$ or ^{b}, $P<0.01^{aa}$ or ^{bb}, $P<0.001^{aaa}$ or ^{bbb}, $P<0.0001^{aaaa}$ or ^{bbbb}

6.5 Discussion

Honey is a potent antibacterial agent eliciting a wide range of effects, these antimicrobial effects have been attributed to the sugar content, H_2O_2 concentration, phenolic compounds, and DB-1.[327] The high sugar concentration creates an osmotic pressure gradient responsible for drawing water out of bacterial cells subsequently inhibiting bacterial cell growth.[328] A unique sugar compound primarily found in Manuka honey, MGO, has been observed to disrupt bacterial structures and morphology ultimately reducing swarming and swimming motility due to de-flagellation and adherence of both Gram-positive and Gram-negative bacterial cultures.[322] Enzymatically produced H_2O_2 has potent antiseptic effects acting as an oxidising agent that disinfects the wound site simultaneously promoting VEGF.[327] Phenolic compounds such as gallic acid and p-coumaric acid exhibit strong anti- and pro-oxidant effects contributing to overall antimicrobial effects.[329] The pro-oxidant effects of phenolic compounds amplifies the activity of H_2O_2 leading to DNA damage of bacterial cells ultimately inhibiting cell growth.[329] DF-1 is an antimicrobial peptide that has a synergistic relationship with H_2O_2 and the osmotic pressure caused by sugar found within honey leads to structural and morphological changes in bacterial cells subsequently disrupting biofilms formed by *S. aureus* and *P. aeruginosa*.[330] For all these compounds, the type and concentration depends on the botanical and geographical origin, bee species, and honey processing.[327] Many of these components act synergistically to increase the potency of honey as an antimicrobial agent against several bacterial strains, including MDR strains.

The UMF is a grading system that measures the bioactive strength and quality of the Manuka honey, quantifying the concentration of essential bioactive compounds that contribute to its antimicrobial effects, for example MGO. This grading system is managed by the UMF Honey Association in New Zealand, ensuring that certified Manuka products align with strict quality standards.[360] The MIC_{50} of the UMF150+ Manuka honey sample against *E. coli* (ATCC 700928) was 4.6 ± 0.6% (v/v), indicating reduced activity compared to the reported MIC_{50} of 2.5% (v/v) for UMF16+ Manuka honey.[331] However, it was more efficacious than UMF25+ Manuka honey, which had an MIC_{50} of 6.25%[332] as identified by Lin *et. al*.[331] and Sherlock *et. al*., [332] respectively, against various *E. coli* isolates from clinical settings. Sherlock *et. al*.[332] prepared bacterial suspensions overnight in nutrient agar, adjusted them to the McFarland standard using sterile saline, and diluted the honey samples through serial double dilution, ranging from 50% to 0.02% (v/v) in nutrient broth, which may account for the differences in the results beyond the antimicrobial activity itself.[332]

Furthermore, the MIC_{50} value of the Manuka honey (UMF 150+) sample used in this study was lower than the 10% (v/v) MIC_{50} determined for Revamil honey against clinical isolates of *E. coli* identified by Kwakman *et. al*.[333] Revamil® honey is a medicinal honey gel responsible for creating a moist wound environment optimal for inhibiting bacterial growth and wound healing.[334] This type of honey exerts its antimicrobial effect through a combination of factors, including high sugar concentration, H_2O_2, MGO, the cationic antimicrobial peptide DF-1, and low pH.[334] This mechanism differs from that of medicinal honeys like Manuka honey, which primarily relies on MGO as its key antibacterial component.[334] MGO disrupts bacterial cell functions by modifying proteins and nucleic acids, ultimately leading to cell death. In contrast, Manuka honey contains negligible levels of H_2O_2 and bee defensin-1, distinguishing it from Revamil® honey. [334] It is important to acknowledge the formulation differences of Revamil® honey and Manuka honey. Revamil® honey is distinct from raw honey due to its standardized formulation, high purity, and controlled composition. As a medicinal-grade honey, it is produced under strictly regulated conditions to minimize bacterial contamination and undergoes gamma irradiation to eliminate spores, including *Clostridium botulinum*. In contrast to raw honey, which can vary in microbial content and chemical composition, Revamil® offers a consistent formulation with a prolonged and elevated release of hydrogen peroxide, enhancing its antimicrobial properties. Additionally, it preserves bee defensin-1, a natural antimicrobial peptide that may degrade over time in raw honey. Given these differences it is interesting to note that the low MIC_{50} value for MA indicates that the raw Manuka honey used in the present study is more effective than Revamil® honey. The BU honey sample exhibited an MIC_{50} of 3.0 ± 0.6% (v/v) against *E. coli* (ATCC 700928), which is lower compared with the MIC_{50} of the Manuka honey (UMF 150+) sample used in this study, UMF 25+ Manuka honey identified by Lin *et. al.*,[331] and Revamil® medicinal honey gel identified by Kwakman *et. al*.[334] Therefore the lower MIC_{50} of the Buckwheat honey sample in this study suggests that Buckwheat honey has superior antibacterial activity against *E. coli* compared to Manuka honeys across various UMF grades as well as Revamil® medicinal honey gel.

Honey samples FB1, FB3, FB6 and FB9 had an MIC_{50} of 7.0 ± 0.2% (v/v), 4.8 ± 0.9% (v/v), 2.6 ± 0.3% (v/v), and 5.3 ± 0.9% (v/v), respectively, against *E. coli* (ATCC 700928). These values are lower than the 25% (v/v) MIC_{50} value of *Erica species* Fynbos honey against *E. coli* identified by Basson and Grobler,[335] and the UMF 25+ Manuka honey with an MIC_{50} of 6.25% (v/v),[332] indicating an improved antimicrobial activity for the FB honeys mentioned previously. The researchers utilised a broth dilution method to assess the antimicrobial properties of the honeys. A 50% (weight/volume) stock solution of each honey was prepared in double-strength sterile Brain Heart Infusion (BHI) broth. This stock solution was then serially diluted to achieve

the desired concentrations for testing. These minor differences in sample preparation may account for the variation in the results beyond the antimicrobial activity itself

Interestingly, all Fynbos samples used in this study had lower MIC_{50} values compared to the 10% (v/v) MIC_{50} value determined for Revamil® medical grade honey against *E. coli* clinical isolates using *in vitro* assessment for the efficacy of reduction of forearm skin colonization, indicating potentially greater activity for some Fynbos honeys compared with Revamil® medical grade sample evaluated by Kwakman *et. al*.[333] Sample FB6 had a MIC_{50} lower than the 4.6 ± 0.6% (v/v) of UMF 150+ Manuka honey used in the present study indicating an improved antimicrobial activity of this FB honey. Sample FB3 had an MIC_{50} value similar to the Manuka (UMF 150+) honey used in the present study indicating a similar activity of this FB honey.

The MIC_{50} of the Manuka honey (UMF 150+) sample was 28.1 ± 6.9% (v/v) against *S. aureus* (DSM 2569), indicating a decrease in antimicrobial activity compared to Medihoney with an MIC_{50} of 1.75% identified by Tirado *et. al.*,[89,336] and Comvita Manuka UMF 25+ with an MIC_{50} of 6.25% (v/v) against *S. aureus* ATCC 43300 identified by Sherlock *et. al.*[89,332] This is possibly related to the high fructose content of the honey samples (Figure 3.4.3), where in a study by Brennan *et. al.*337 and Garnett *et. al.*[338] an increase in *S. aureus* growth was observed in the presence of a high sugar environment.[337,338] However strain differences may also account for differences against *S. aureus,* the study conducted by Tirado *et. al.*[336] and Sherlock *et. al.*[332] were *in vitro* analysis using the broth microdilution assay. Tirado *et. al.*[336] used Colombia Blood Agar and it was unclear the agar used in Sherlock *et. al*.[332] These differences in the type of agar result in different nutrient availability, standard M-H broth can contain variable cation concentrations dependent on the batch or brand used, influencing susceptibility outcomes resulting in discrepancies in susceptibility assessments.[339,340]

Samples FB1, FB3, FB6 and FB9 had an MIC_{50} of 0.7 ± 0.2% (v/v), 39.2 ± 15.6% (v/v), 31.7 ± 11.0% (v/v), and 0.4 ± 0.1% (v/v), respectively, against *S. aureus* (DSM 2569). Samples FB3 and FB6 had a lower activity compared with 28.1 ± 6.9% (v/v) for UMF 150+ Manuka honey although differences are not statistically significant. Compared with Medihoney with an MIC of 1.75% against *S. aureus* (ATCC 43300)89,336 and Comvita Manuka UMF 25+ with an MIC_{50} of 6.25% (v/v) against *S. aureus* (ATCC 43300)89,332 the activity of the FB honey against this bacterium was much lower. The antibacterial activity of FB1 and FB9 is higher, compared with Comvita Manuka UMF 25+ with an MIC_{50} of 6.25% (v/v) against *S. aureus* (ATCC 43300),[89,332] the 25% (v/v) MIC for Erica species Fynbos honey against *S.*

aureus (NCTC 8530)[335] and 28.07 ± 6.85% (v/v) for UMF 150+ Manuka honey in the present study. Honey FB2 had a MIC_{50} of >50% (v/v) for both the *E. coli* (ATCC 700928) and *S. aureus* (DSM 2569) strains, indicating a lack of antibacterial activity.

The MIC_{50} values of the protein fractions was 0.36 ± 0.06 mg/mL for MA, >0.11 mg/mL for BU, 0.11 ± 0.04 mg/mL for MF1, and for Fynbos honey the range was >0.01 - 0.13 ± 0.04 mg/mL against *E. coli* (ATCC 700928). Against *S. aureus* (DSM 2569) the MIC_{50} values were 0.14 ± 0.08 mg/mL for MA, >0.11 mg/mL for BU, 0.01 ± 0.01 mg/mL for MF1 and the range for the Fynbos protein fractions were 0.05 ± 0.04 - >0.10 mg/mL for the FB samples, and against *S. aureus* (DSM 2569), (Table 6.4.1).

DF-1 effectively inhibits Gram-positive bacteria (*S. aureus* (DSM 2569)),[245] although it is a common component within honey protein, concentrations are variable and correlates with antibacterial activity against *S. aureus*.[245] The low MIC_{50} values MA, FB1, FB2, and MF1 protein fractions is possibly linked to the presence of DF-1 in these honey samples however the this would need to be confirmed with LC-MS/MS analysis. Bucekova *et. al.*[245] reported DF-1 in honey at 5-10% dilution, does not contribute significantly to antibacterial activity against *P. aeruginosa* (CCM 1960) and *S. aureus* (CCM 4223) and therefore for the dilutions used in this study may not have a significant effect.

The antimicrobial activity of many honeys is due to a peroxide-based mechanism from the relationship between bee derived glucose oxidase and H_2O_2. Hydrogen peroxide has potent antiseptic effects acting as an oxidising agent that disinfects the wound site simultaneously promoting VEGF.[327]

There was an observed increase in susceptibility of Gram-negative *E. coli* (ATCC 700928) cultures when treated with whole honey samples, and an observed increase in susceptibility of Gram-positive *S. aureus* (DSM 2569) cultures when treated with the protein isolates. In this study, Fynbos honey has been identified to have antibacterial activity and differences in the MIC_{50} potentially related to the botanical origin, geographical origin, season, bee health, and processing of the honey samples.[144]

6.6 Conclusion

This study identifies that the antibacterial activity of Fynbos honey, except for FB2 is similar to MA and BU. The low MIC_{50} values associated with most of the Fynbos protein fractions is possibly due to the presence of DF-1, although confirmation with LC-MS/MS analysis is

required. A limitation of MIC_{50} determination is that it is only an indicator of activity and does not identify if the effects are bacteriostatic or bactericidal.

Chapter 7: Concluding discussion

Chronic wounds are difficult to treat and with the emergence of multidrug resistance, the number of drugs for the treatment of the associated infections are limited. Increasingly medicinal honeys such as Manuka, Buckwheat and Revamil® honey are being used in the treatment of chronic wounds. Not only are the infections eradicated, but other processes in wound healing are targeted, such as the inflammatory phase and the process of re-epithelisation. Although effective, these honeys are not locally available and import costs are high. In an endeavour to develop South African honeys as an alternative therapy, it is necessary to evaluate several properties of these honeys related to wound healing. This will provide information required for further animal-based studies.

Several characteristic features contributed to the unique properties of certain types of medicinal honey, for Manuka honey it is the presence of methylglyoxal (MGO) at very high concentrations,334 Buckwheat with high H_2O_2 levels[106] and Revami®l honey (made from RS honey) due to high concentrations of DF-1, a cationic antimicrobial peptide.334

The protein component of South African Fynbos honey is a widely under researched topic. The protein components of other medicinal honeys have been researched where it was found that the MRJP family of proteins function in anti-inflammatory activity, specifically MRJP 1, MRJP 2, and MRJP 3. MRJP 2 have shown their ability to stimulate tumour necrosis factor alpha (TNF-α) and immunoglobulin E, whilst MRJP 3 has been observed to suppress pro-inflammatory cytokines in mice models.[298–301] Furthermore MRJP 3, has shown anti-inflammatory function by supressing the release of IL-2, IL-4, and interferon-γ.[142] Antibacterial activity is also associated with GOx, this enzyme catalyses the oxidation of glucose and H_2O to gluconic acid and H_2O_2.[129,144] H_2O_2 has been described as an oxidative biocide,[144] sterilizing the wound by eliciting oxidative damage to microorganisms through interaction with the bacterial cell wall, intracellular lipids, proteins, and nucleic acids.[109]

This purpose this research is to bridge the gap in knowledge, by exploring the associated bioactivity of the honeys and the associated protein fraction of Southern African honey. Thus, the aim of this study was to determine the bioactivity of Fynbos honey and the associated bioactivity of the protein fraction.

7.1 Summary of results

Eight honey samples were utilised for this investigation with their respective isolated protein fractions, compared to Manuka and Buckwheat honeys, acting as controls. Protein fractions

were isolated using gel filtration chromatography, adapted from Erban *et. al.*,[7] from 25% (v/v) aqueous solutions of Manuka and Buckwheat (controls) and five Fynbos honeys (FB1, FB2, FB3, FB6 and FB9).

In Chapter 3, physiochemical properties of the honeys were determined, and the protein fraction was isolated and characterised. Conditions were selected to obtain a protein fraction free from smaller molecules such as sugars, polyphenols and H_2O_2.

The protein fractions did not represent the entire protein content but was representative of the proteins found in these honeys. The fractions with the highest concentration of sugar were eluted after the protein fractions, there was no observed overlap between the concentrated sugar fractions and the concentrated protein fractions. The difference in elution of the proteins and sugars indicates the effective separation of the sugar and protein content within the samples, using gel filtration chromatography.

The proteins tentatively identified with SDS-PAGE were the proteins of the MRJP family and possibly DF-1 being present in several Fynbos samples. Both the MRJP family with addition DF-1 are associated with antimicrobial activity, these peptides function in the innate immune response of the bee, eliciting an antimicrobial effect against fungi, yeast, protozoa, Gram-positive and Gram-negative bacteria. DF-1 has an antimicrobial function by creating a pore or breakage in the cell membrane, disrupting membrane permeability, resulting in cell death.[118] A study conducted by Brudzynski and Sjaarda[143] identified two distinctive antimicrobial functions of isolated GP samples containing MRJP 1 and associated jellein peptides these were specific binding and agglutination of bacterial cells, and non-specific membrane permeabilization of bacterial cells.[143]

The efficacy of the isolation method and consolidation of protein concentration was confirmed through the similarity of intensive protein bands for both the honey samples and protein fractions indicating a similarity of predominant proteins ranging from 45-70 kDa following isolation. These bands are hypothesised to be a part of the MRJP protein family, and GOx, aligning with the findings of Lewkowski *et. al.*,[156] Paget *et. al.*,[205] and Bucekova *et. al.*[140] Furthermore, the similarity in the protein patterns of the Fynbos samples and Manuka control indicates a crossover of protein content between the South African honey and medical grade honey. Therefore, in chapter 3 it was determined that the South African Fynbos honeys have a similar physiochemical makeup when analysed as a whole honey solution and following protein isolation, the implications of this physiochemical similarity are a potential similarity in

cell migratory, antioxidant, anti-inflammatory, and antimicrobial bioactivity. Where South African Fynbos honey could prove to be investigated as a potential medical grade honey.

Reepithelialisation and the establishment of functional dermis is an important phase of wound healing. In chapter 4, the cytotoxicity and the cell migratory effects of the honeys and the protein fractions. This was achieved using the and cell migratory effects of the honey samples and protein isolates was tested on a human keratinocyte (HaCaT) and murine fibroblast (SC-1) cell line as both cell types are key cellular role players in wound healing that synergistically respond to paracrine signals from one another to aid in correct wound healing and closure. For the cell migration studies, the antioxidant and anti-inflammatory studies, a low non-cytotoxic concentration was required. With the SRB cytotoxicity assay, the cell viability of the HaCaT and SC-1 cell line. The cell viability for the honey samples at 1.25% (v/v) (HaCaT cell model) and 0.625% (v/v) (SC-1 cell model) and (0.044-0.146 mg/g) protein fractions (HaCaT and SC-1 cell models) was > 80% and according to the ISO 10993-5 cell viability criteria are non-cytotoxic.[233]

The wound closure rates of the Manuka and Buckwheat honey and protein controls, and several Fynbos honey samples and protein fractions using the HaCaT cell model. Compared with the study of Martinotti *et. al*.[247] wound closure in the present study was better than that reported for 0.1% Manuka honey on HaCaT cells. The wound closure rate of the Fynbos honey samples, and protein fractions was not significantly different to the Manuka and Buckwheat honey. Likewise, the wound closure of the protein fractions was also similar to the honey samples, indicating that the proteins in honey contribute to wound healing

The wound closure rate of the honey samples at 1.25% (v/v) (HaCaT cell model), 0.625% (v/v) (SC-1 cell model) and protein fractions (HaCaT and SC-1 cell models) was stabilised across both cell lines. A study conducted by Bucekova *et. al.*[139] revealed a dose dependent relationship between DF-1 and MMP-9 secretion, subsequently promoting the migration of keratinocytes *in vitro*.[139] This dose dependent relationship identified by Bucekova *et. al*.[139] could explain the observed stabilisation of the wound closure rates of the protein isolates, the protein fractions do not experience a significant loss of wound closure activity in comparison to their whole honey counterparts.

In chronic wounds the inflammatory phase is prolonged and effective reduction of mediators of inflammation is beneficial. In Chapter 5, the antioxidant properties of the honey and the protein fractions was determined. The antioxidant capacity of the Fynbos honey samples,

measured using ORAC and TEAC assays, showed moderate activity compared to other Fynbos and Manuka (UMF 22+ and UMF 150+) honeys identified by Baretta *et. al.,*[281] Anand *et. al.*,[279] Yusof *et. al.*,[280] and interobserver studies conducted by Serem and Bester[152] and Magoshi *et. al*.[199] ORAC values ranged between 1.79 ± 0.41 to 2.67 ± 0.73 µM TE/g, lower than those reported for Manuka (48.41 ± 14.1 µM TE/g) and other Fynbos honeys. Similarly, TEAC values for the Fynbos honey samples (2.05 ± 0.35 to 6.27 ± 0.46 µM TE/g) were generally lower than or comparable to other studies, with only a few samples showing matching activity. In both assays, the antioxidant capacity of the Fynbos protein fractions was minimal and consistently lower than that of other honey types and grades analysed in previous studies. These results indicate that while Fynbos honeys offer some antioxidant benefits, they exhibit reduced activity in comparison to Manuka and medical grade honeys, with the protein extracts showing limited antioxidant potential. To further consolidate these findings the protective capacity of the honey samples and protein fractions from AAPH generated peroxyl radical damage on HaCaT and SC-1 cells were analysed using the DCFH-DA assay, The oxidative damage on HaCaT cells for all samples was >80%, indicating a poor antioxidant capacity. Interestingly, all Fynbos protein fractions, and the MF protein fraction had an OD >100% possibly indicating a pro-oxidant effect of the protein isolates. Moreover, the antioxidant capacity seems to be improved on the SC-1 cells whose %OD was less than that identified for the HaCaT cells.

Interestingly, the acellular anti-inflammatory activity of the Fynbos protein isolates is greater than that of the Fynbos honey samples analysed in this research. The implication of this is the acellular NO scavenging of the protein fractions is increased in comparison to that of their honey counterparts however, this data was not consolidated in the *in vitro* anti-inflammatory activity using the +LPS/RAW 264.7 cell model. The anti- and pro-inflammatory activity related to NO scavenging and induction were subsequently The NO scavenging ability of 1.25% (v/v) Fynbos honey was 2.53± 0.61-8.78± 2.90 µM (refer to figure 5.4.2.2a). The NO concentration determined by Larsen *et. al.*[294] for the medical grade Canadian honey lozenge and drop formulations was higher compared to that produced in this current study which utilised the same *in vitro* model. Thereby suggesting an improved anti-inflammatory potential of the Fynbos honey samples used in this research compared to the medical grade Canadian honey lozenge and drop formulations analysed by Larsen *et. al*.[294]

An important aspect in the healing of chronic wounds is the eradication of infection, and finally in Chapter 6, the MIC values for the honeys and protein fractions was determined against a model Gram-positive and -negative bacteria, *E. coli* (ATCC 700928) and *S. aureus* (DSM

2569) respectively. The MIC_{50} values of the Fynbos honey samples and protein fractions against *E. coli* (ATCC 700928) were, lower than MIC_{50} value of *Erica species* Fynbos honey against *E. coli* (NCTC 9001) identified by Basson and Grobler.[335] indicating an improved antibacterial activity of the Fynbos samples used in this study. Furthermore, the fynbos honey samples used in this study had improved antibacterial activity in comparison to a UMF 25+ Manuka honey identified by Sherlock *et. al.*,[332] and had lower MIC_{50} values compared that determined for Revamil® medical grade honey against clinical isolates of *E. coli* suggesting an increased antibacterial activity of the Fynbos honey samples in comparison to the Revamil® medical grade sample identified by Kwakman *et. al*.[333] Selective Fynbos samples had an observed increase in antimicrobial activity compared to Comvita Manuka UMF 25+ against *S. aureus* (ATCC 43300),[89,332] and the *Erica species* Fynbos honey against *S. aureus* (NCTC 8530) identified by Basson and Grobler.[335] Furthermore, this study determined improved susceptibility of Gram-negative *E. coli* (ATCC 700928) cultures to the honey samples, aligning with the findings of Escuredo *et. al.*,[318] Isla *et. al.*,[319] Fyfe *et. al.*,[320] and Mohapatra *et. al.*,[321] and Gram-positive S. aureus (DSM 2569) cultures to the protein isolates. The observed antibacterial activity of the Fynbos honey samples, and protein fractions were, in some cases, higher than that of Manuka honey across several UMF grades. Indicating a similarity and somewhat improved activity in comparison to the Manuka honey golden standard.

In conclusion, this research has determined parallels between the molecular mass patterns analysed using SDS-PAGE, *in vitro* anti-inflammatory potential using the +LPS/RAW 264.7 cell model, and antibacterial activity against *E. coli* (ATCC 700928) and *S. aureus* (DSM 2569) analysed using broth microdilution determining MIC_{50}'s of South African Fynbos honey samples and Manuka honey or medical grade honey samples/products. Although, the Fynbos honey samples did not have a strong antioxidant capacity, the samples had a similar antioxidant capacity to other South African Fynbos honey samples, or Manuka honey, drawing parallels in antioxidant activity. This study was able to further elucidate the potential anti- and pro-inflammatory activity of the protein isolates using the +LPS- and -LPS/RAW 264.7 cell model, cell migratory activity stabilisation, and antibacterial activity against *E. coli* (ATCC 700928) and *S. aureus* (DSM 2569) due to the presence of hypothesised DF-1 (determined using SDS-PAGE) of protein isolates from honey.

7.2 Limitations and recommendations

Although this research provides valuable insights, the limitations must be acknowledged. The column fractions collected and pooled do not represent the entire protein fraction and, in

several instances, direct correlation between the honeys and the protein fractions could not be determined, therefore making comparison became a challenge. In future studies, further optimisation of this technique by either running multiple isolations using individual fractions, and/or assessing the difference between chromatography column lengths and isolation be undertaken.

In addition, the use of colorimetric indicators, such as the spot tests used in this study, relies heavily on visual assessment, which could be prone to subjectivity or inconsistency. The intensity of colour can vary due to factors such as the concentration of the substances or the time taken for the reaction, making it difficult to standardise results without proper controls or quantification technique. The lack of an interobserver control for spot tests used in this study has limited the accuracy in protein retention following isolation. Therefore, for future studies, using a more sophisticated identification and quantification system such as an Orbitrap Fusion Tribrid mass spectrometer coupled with gel filtration chromatography to accurately detect and quantify physiochemical properties in the isolates.

Although SDS-PAGE is a widely used and understood technique in protein, limitations of this technique include the occurrence of proteolytic degradation during sample preparation with a resultant loss of protein bands or poor resolution.[209] Additionally, the bands on the SDS-PAGE gels are representative of multiple proteins at an estimated molecular weight (kDa). Due to this, identification of responsible proteins for subsequent activities analysed was not specific and therefore drawing conclusions with regards to how the proteins had an improved activity was not possible. For future studies, exploring honey fingerprinting using liquid chromatography mass spectrometry (LC-MS/MS) analysis[210] against known proteins for *A. mellifera* to accurately determine and confirm the presence of the MRJP protein family, GOx and/or DF-1 within the honey samples and protein fractions. This fingerprinting will allow for increased accuracy when pooling protein fractions following isolation ultimately increasing the protein yield and mitigating the loss of protein and peptide content. Furthermore, honey fingerprinting will improve reporting on specific proteins responsible for observed antioxidant, anti-inflammatory, and anti-microbial activity.

While in *vitro* cytotoxicity analysis is crucial, limitations do exist, such as the inability to accurately predict the *in vivo* response. Consequently, a combination of *in vitro* and *in vivo* analysis is essential in comprehensive screening of safety and efficacy.[242] While *in vitro* analysis of wound closure through scratch migration assays is useful in examining cell migration under standardised conditions enhancing reproducibility it is important to

acknowledge the limitation of this technique.[341] Limitations of this scratch assay technique include, the manual scratching of the cell monolayers allowing for variation in the size and shape of the scratches, accumulation of cellular debris on the edge of these scratches, as well as inaccurate reporting of cell migration leading to false positives.[219] Various methods have been described and include the use of serum starvation preceding the scratch and/or the use of less serum in media. Nonetheless in the present study, this assay does show the potential of honey and the protein fraction to promote cell migration. Therefore, it is recommended for future studies to use a transwell migration assay to accurately distinguish migration from proliferation. In addition, analysis of the biochemical markers associated with migration can provide more insight in the pathways involved. In addition, an *in vivo* wound model can provide key information, in a three-dimensional environment of FB honey and the protein fractions on cell migration.

The increased acellular anti-inflammatory and *in vitro* pro-inflammatory activity of the Fynbos protein isolates is an early-stage discovery with a limited samples size which may limit the generalisation of findings. To conclusively identify these properties of FB honey more data across a larger batch of honey samples from various geographical origins to fully elucidate the extent of this anti-inflammatory activity for clinical applications. Honey fingerprinting with LC-MS/MS MALDITOF analysis[210] against known proteins for *A. mellifera*, would be beneficial in determining the components responsible for the observed increase in acellular anti-inflammatory and *in vitro* pro-inflammatory activity of the protein fractions. As this is a relatively new finding, replicate studies investigating this increased acellular anti- and *in vitro* pro-inflammatory activity should be completed using analytical techniques such as enzyme-linked immunosorbent assays and reverse transcription polymerase chain reaction assays[305] to allow for quantification of specific biomarkers such as IL-1β and TNF-α providing key insight into the inflammatory response triggered by honey protein isolates.

In this study, initial screening for antibacterial activity was against model Gram-positive and -negative pathogens was undertaken. Where clear differences in activity was observed for different FB samples, when the honey and the protein fractions were compared. Further testing should include bacterial strains that often infect chronic wounds such as *Staphylococcus epidermidis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, and Proteus mirabilis*,[342,343] as well as MDR bacterial strains, methicillin-resistant *S. aureus*, vancomycin-resistant enterococci, *Acinetobacter calcoaceticus-baumannii* complex.[23,344] Inclusion of these various bacterial strains will provide a more comprehensive analysis of clinically relevant bacterial strains infecting chronic

wounds. In addition, the determination of MIC_{50} values only may not fully elucidate the dynamic nature of bacterial populations or the pharmacokinetics of antimicrobials *in vivo*.[345] Therefore, while MIC_{50} values provide useful insights, that should be interpreted alongside a broader context of clinical and microbiological data, such as minimum bactericidal concentrations, to navigate effective treatment strategies.

It would be beneficial to investigate the combination of *in vitro* and *in vivo* analysis for a comprehensive screening of safety and efficacy, whereby, including the complementary use of the *in vivo Galleria mellonella* burn models and/or porcine puncture wound models to observe cell viability and cell migratory effects in a more complex cellular environment.

Therefore, this research has aided in the understanding of the physiochemical properties, cell migratory effects, antioxidant, anti-inflammatory, and antimicrobial activity of South African Fynbos honey and its protein isolates. These findings established a framework for future research and practical applications, allowing for continued exploration. This study determined, the wound closure rate, simulated in the scratch migration assay, of the Fynbos samples was not significantly different to the Manuka and Buckwheat controls indicating a similarity in activity of the South African honeys to the medical grade controls, the oxidative damage on HaCaT cells determined using the DCFH-DA assay for all samples was >80%, indicating a poor cellular antioxidant capacity. Interestingly, all Fynbos protein fractions and the MF1 protein fraction had an OD > 100% possibly indicating a pro-oxidant effect of the protein isolates. Moreover, the cellular antioxidant capacity seems to be improved in the SC-1 cells whose %OD was less than that identified for the HaCaT cells. Furthermore, the direct NO scavenging by the protein fractions is greater than the honey samples but was not observed using an *in vitro* model of anti-inflammatory activity and in contrast a pro-inflammatory effect was observed, this research highlights the complexity of antioxidant and inflammatory mechanisms of honey where further characterisation of the proteins involved, and pathway elucidation is required. And improved susceptibility of the honey samples on Gram-negative *E. coli* (700928) cultures and protein isolates on Gram-positive *S. aureus* (DSM 2569) cultures.

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Chapter 9: Appendices

9.1 Appendix A: Chapter 3 supplementary data

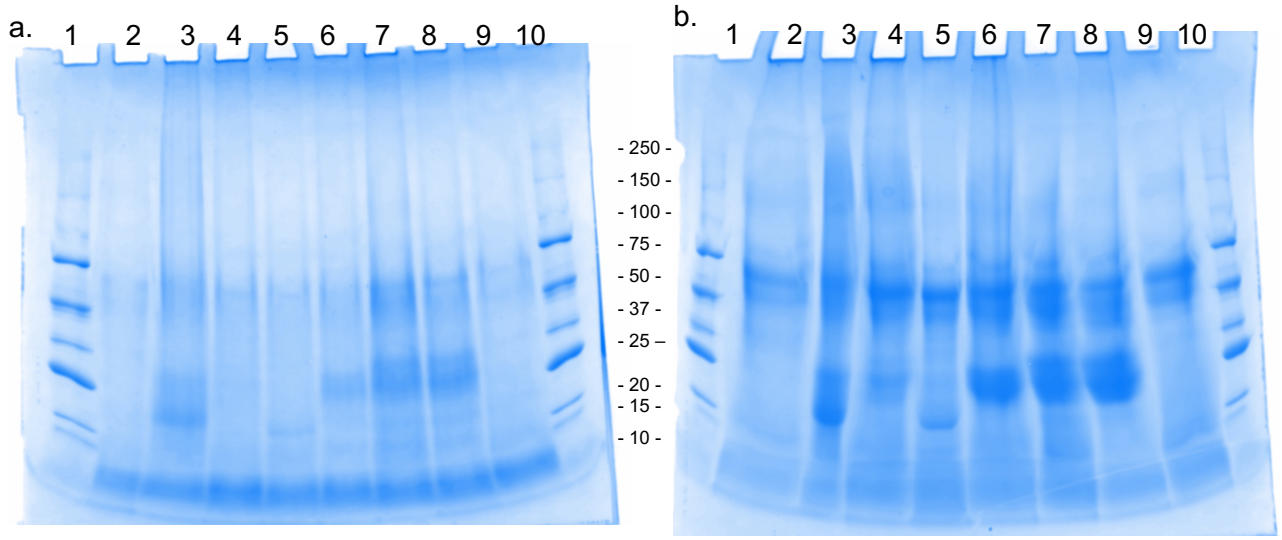


Figure 9.1.1. Replicate 1 of a 4-20% SDS-PAGE Tris Glysine gel indicating the (a) 15 µg whole honey and (b) 2 µg protein and peptide mass profiles of each sample. MA (lane 2), BU (lane 3), FB1 (lane 4), FB2 (lane 5), FB3 (lane 6), FB6 (lane 7), FB9 (lane 8), and MF1 (lane 9) in comparison to a Precision Plus ProteinTM unstained standard (Lasec, BioRad, South Africa) (Lanes 1 and 10) for both (a) and (b).

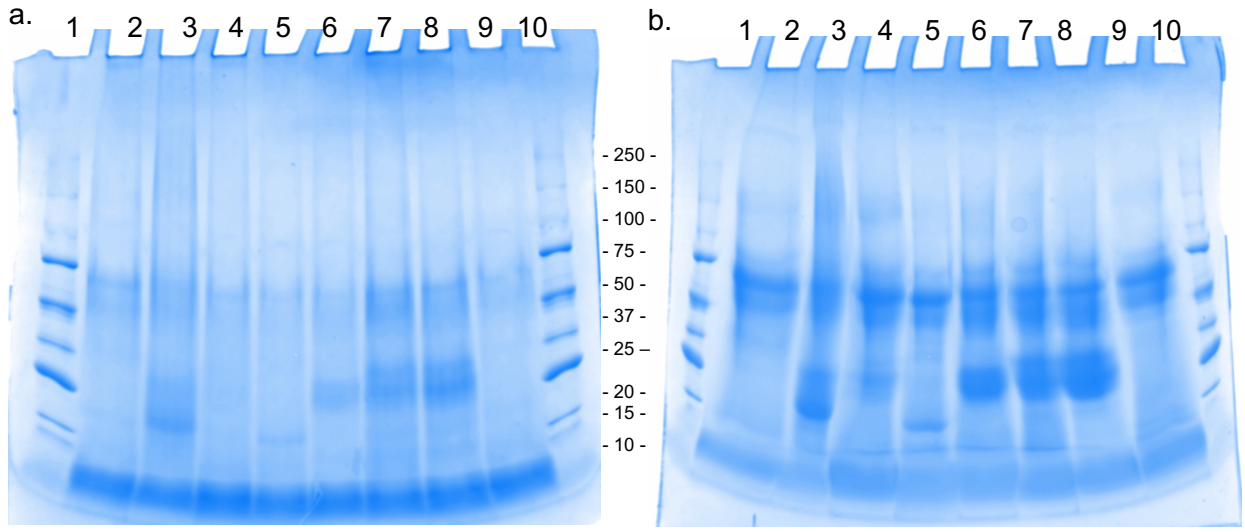


Figure 9.1.2. Replicate 2 of a 4-20% SDS-PAGE Tris Glysine gel indicating the (a) 15 µg whole honey and (b) 2 µg protein and peptide mass profiles of each sample. MA (lane 2), BU (lane 3), FB1 (lane 4), FB2 (lane 5), FB3 (lane 6), FB6 (lane 7), FB9 (lane 8), and MF1 (lane 9) in comparison to a Precision Plus ProteinTM unstained standard (Lasec, BioRad, South Africa) (Lanes 1 and 10) for both (a) and (b).

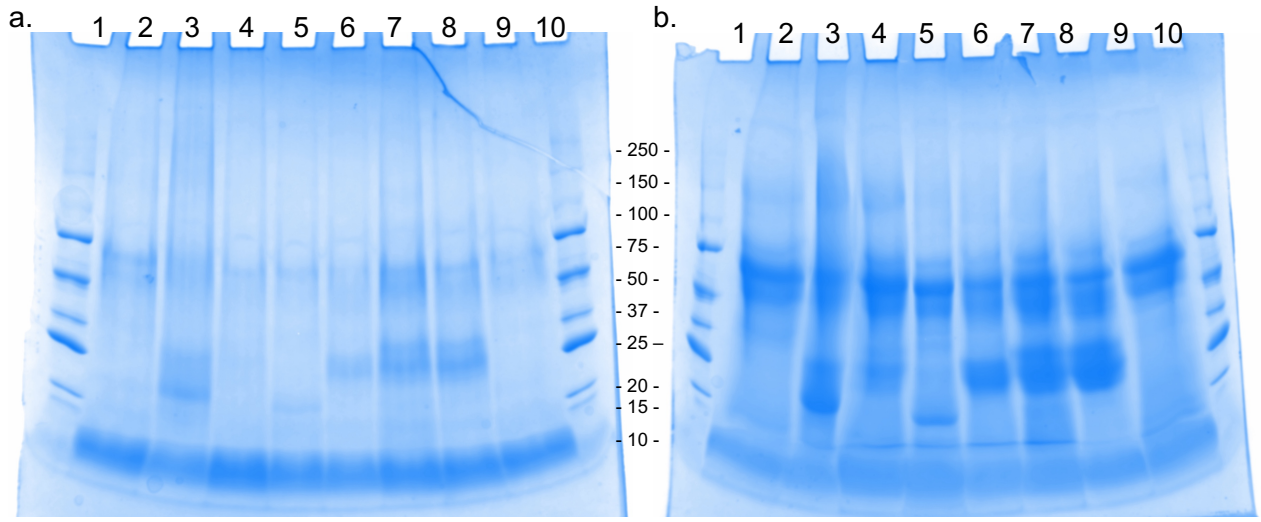
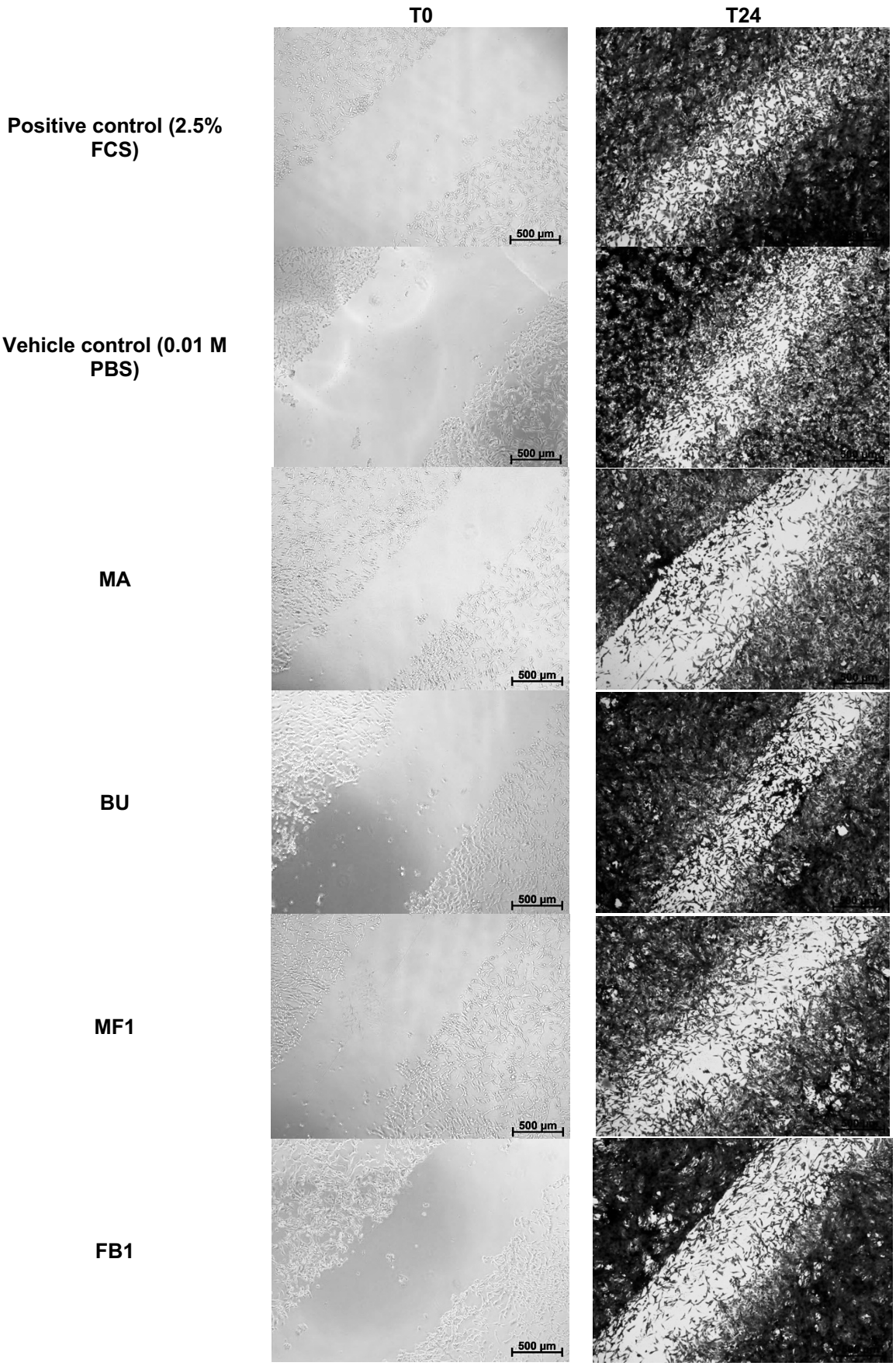


Figure 9.1.3. Replicate 3 of a 4-20% SDS-PAGE Tris Glysine gel indicating the (a) 15 µg whole honey and (b) 2 µg protein and peptide mass profiles of each sample. MA (lane 2), BU (lane 3), FB1 (lane 4), FB2 (lane 5), FB3 (lane 6), FB6 (lane 7), FB9 (lane 8), and MF1 (lane 9) in comparison to a Precision Plus Protein™ unstained standard (Lasec, BioRad, South Africa) (Lanes 1 and 10) for both (a) and (b).

9.2 Appendix B: Chapter 4 supplementary data



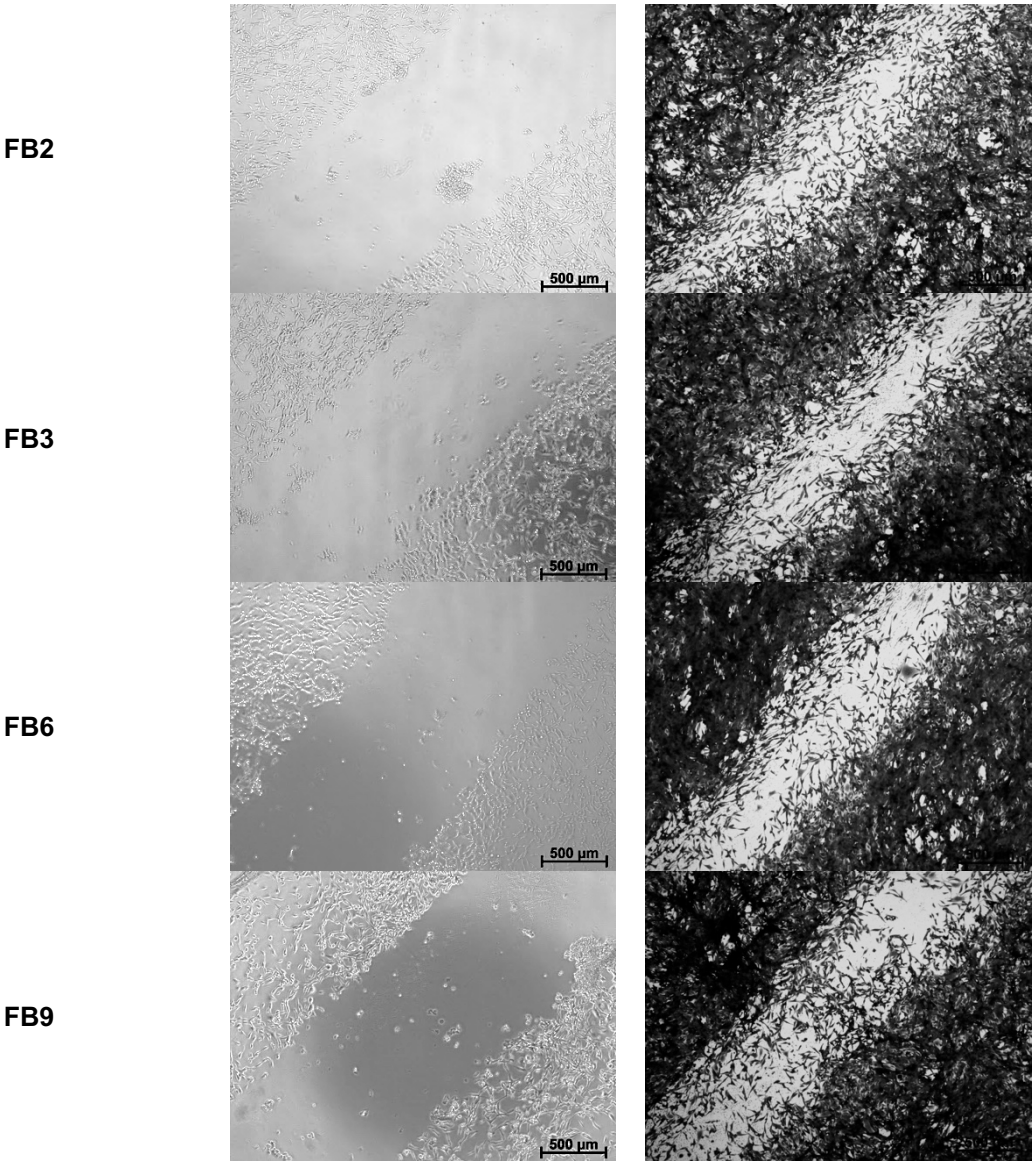
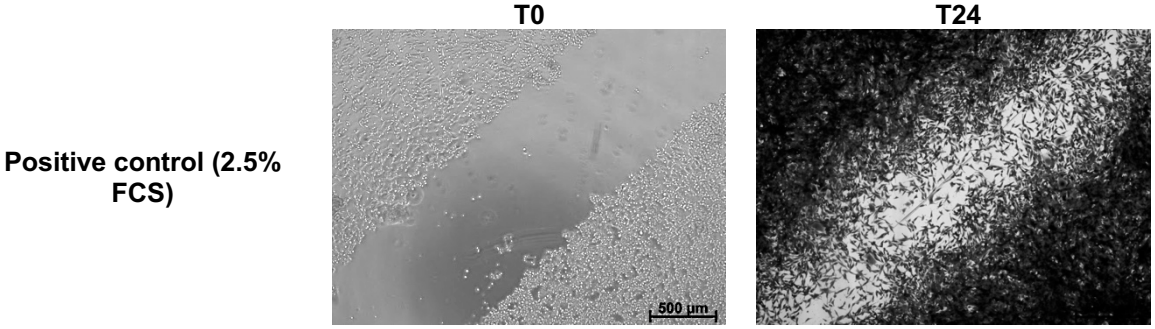
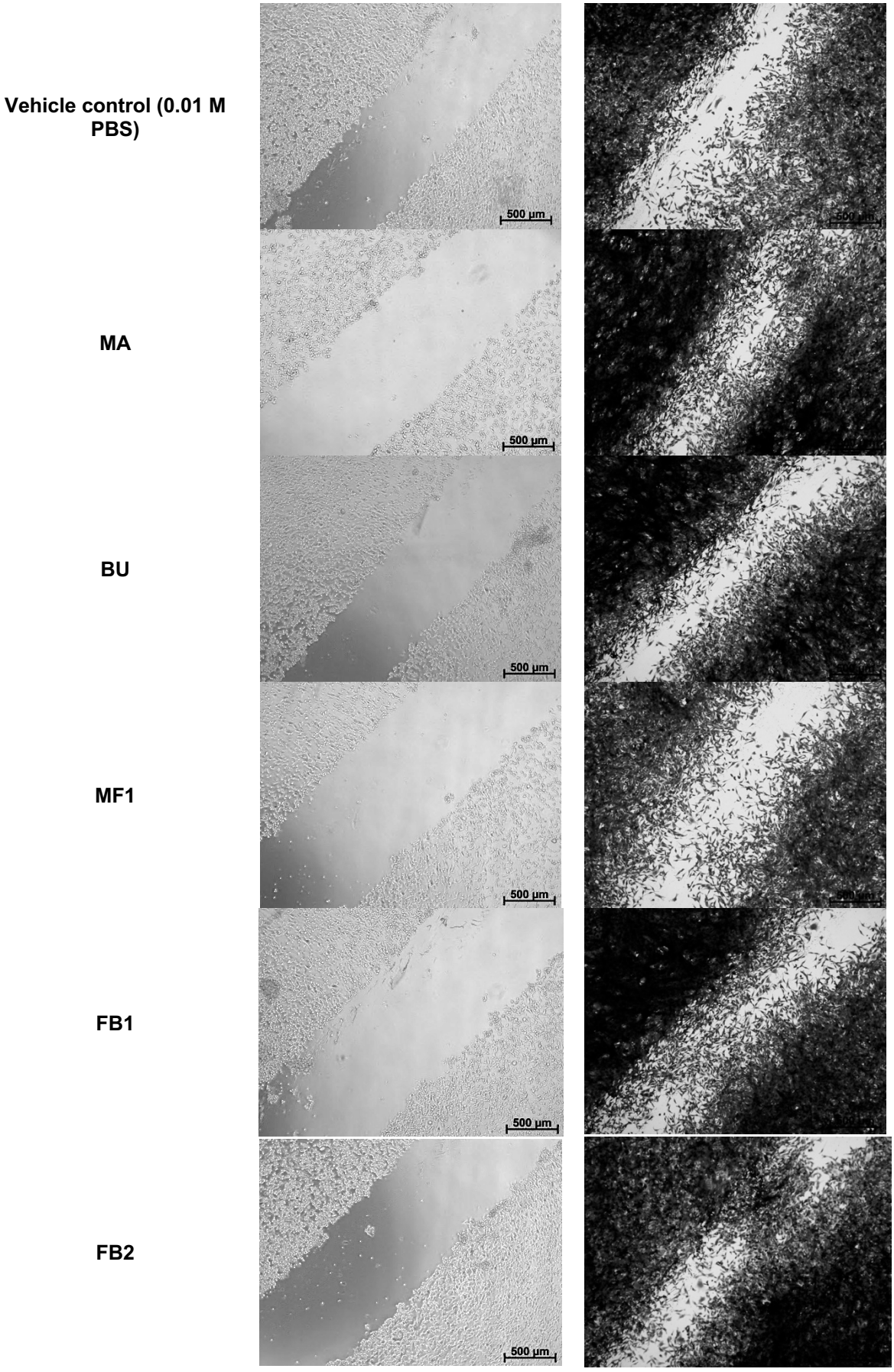


Figure 9.2.1. The cell migration of 1.25% (v/v) whole honey samples assessed using the scratch migration/HaCaT cell model replicate 1. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.





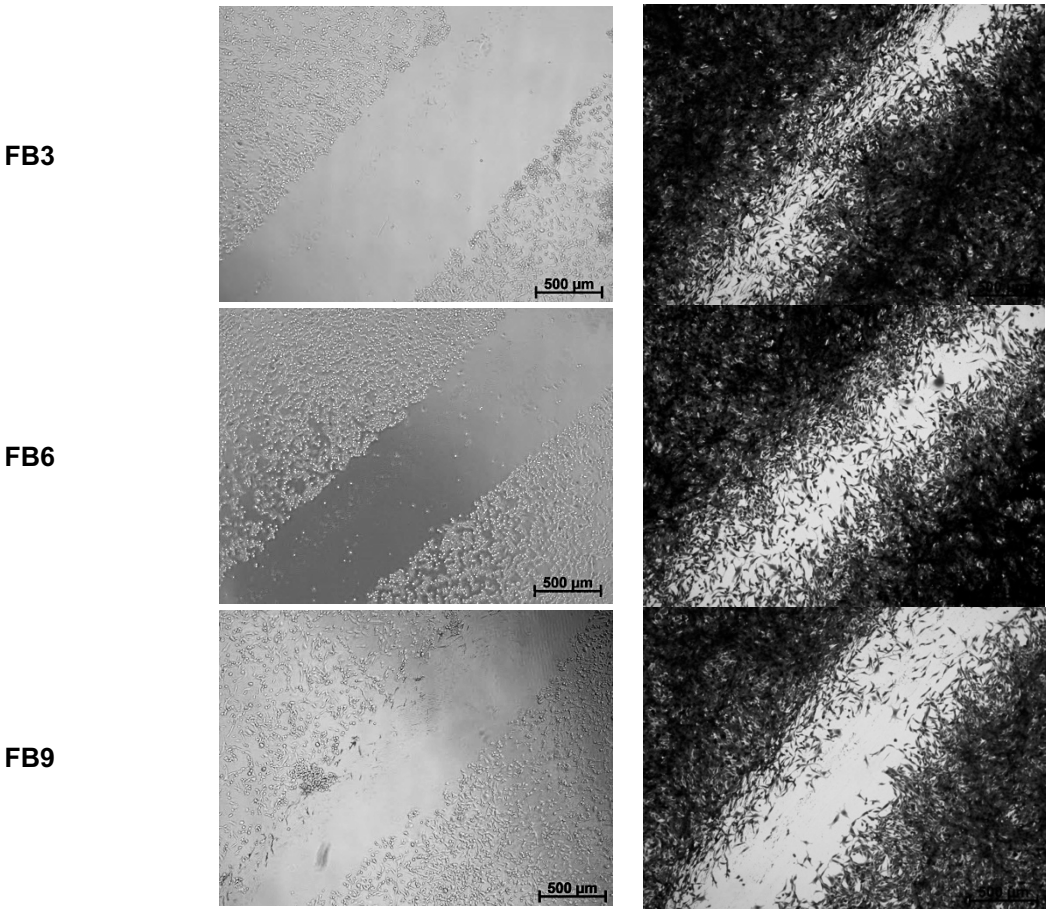
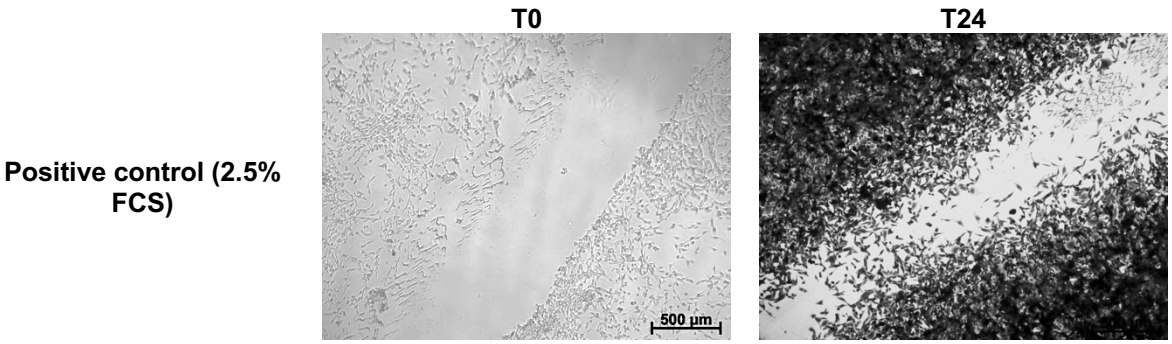
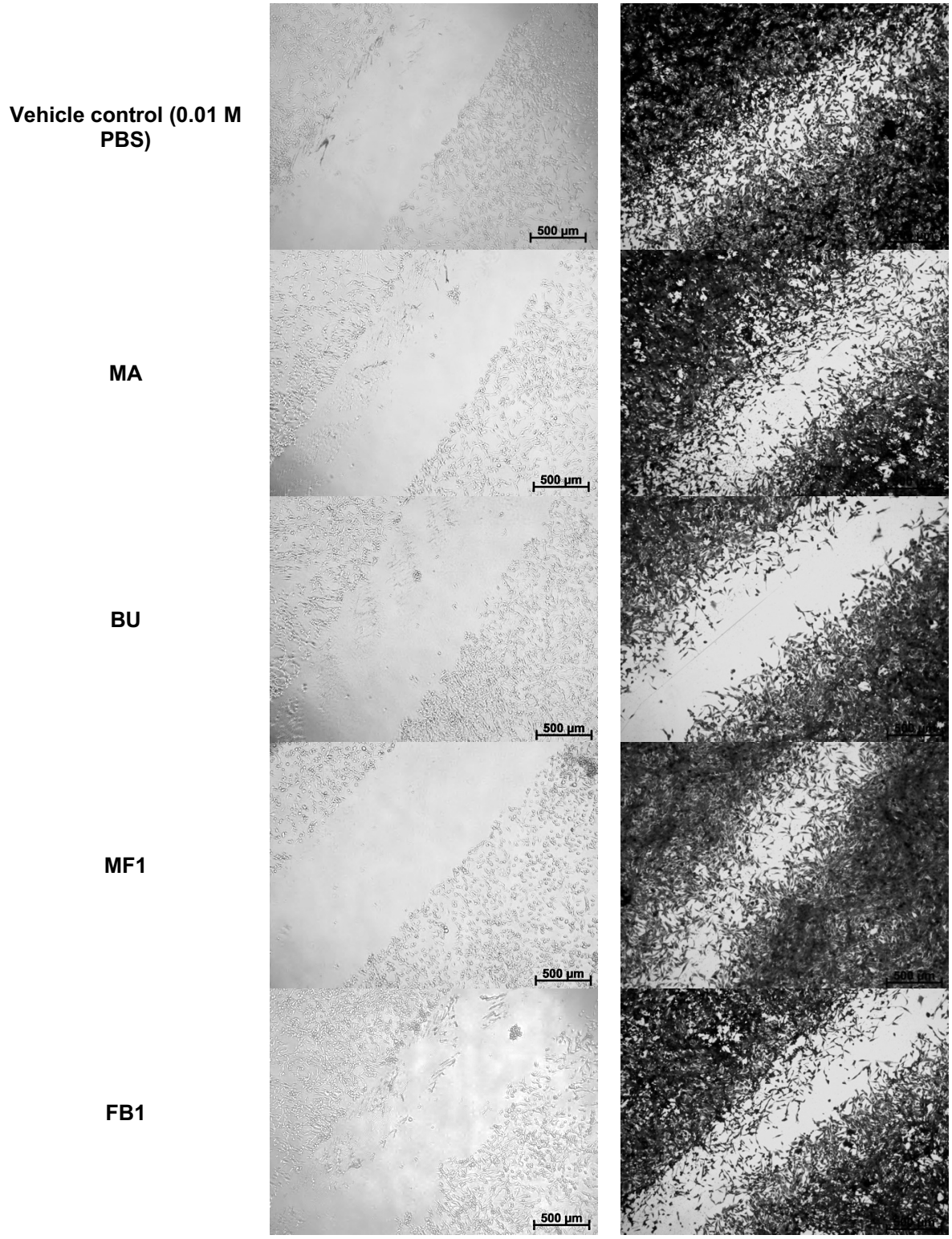


Figure 9.2.2. The cell migration of 1.25% (v/v) whole honey samples assessed using the scratch migration/HaCaT cell model replicate 2. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.





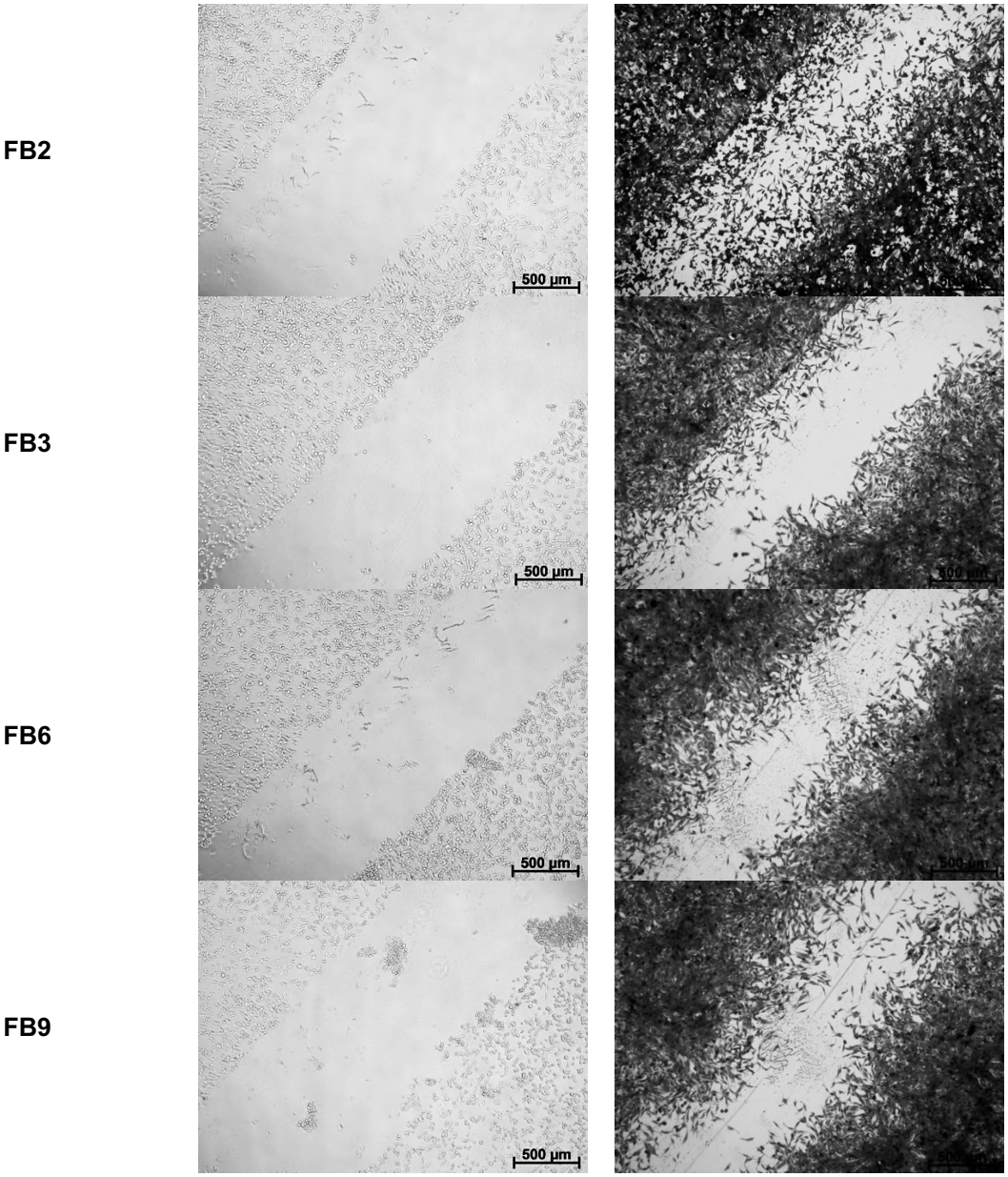
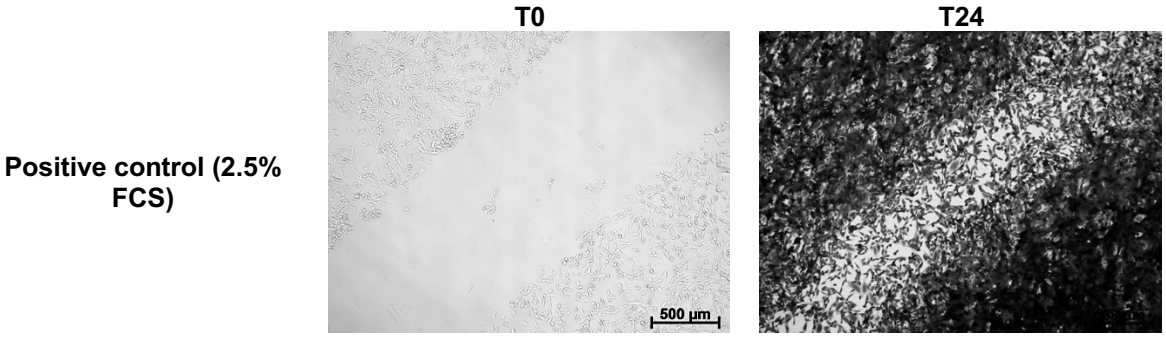
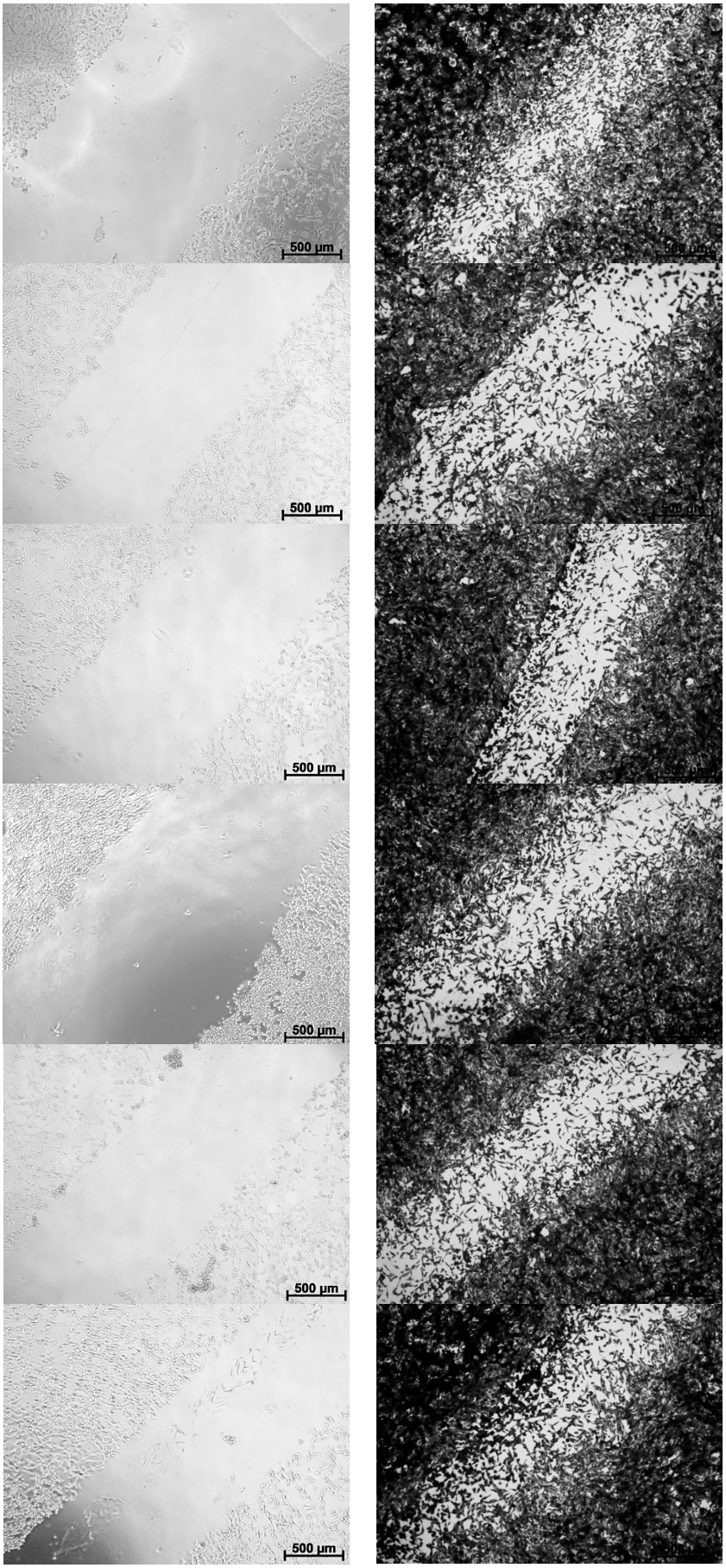


Figure 9.2.3. The cell migration of 1.25% (v/v) whole honey samples assessed using the scratch migration/HaCaT cell model replicate 3. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.



Vehicle control (0.01 M PBS)



MA

BU

MF1

FB1

FB2

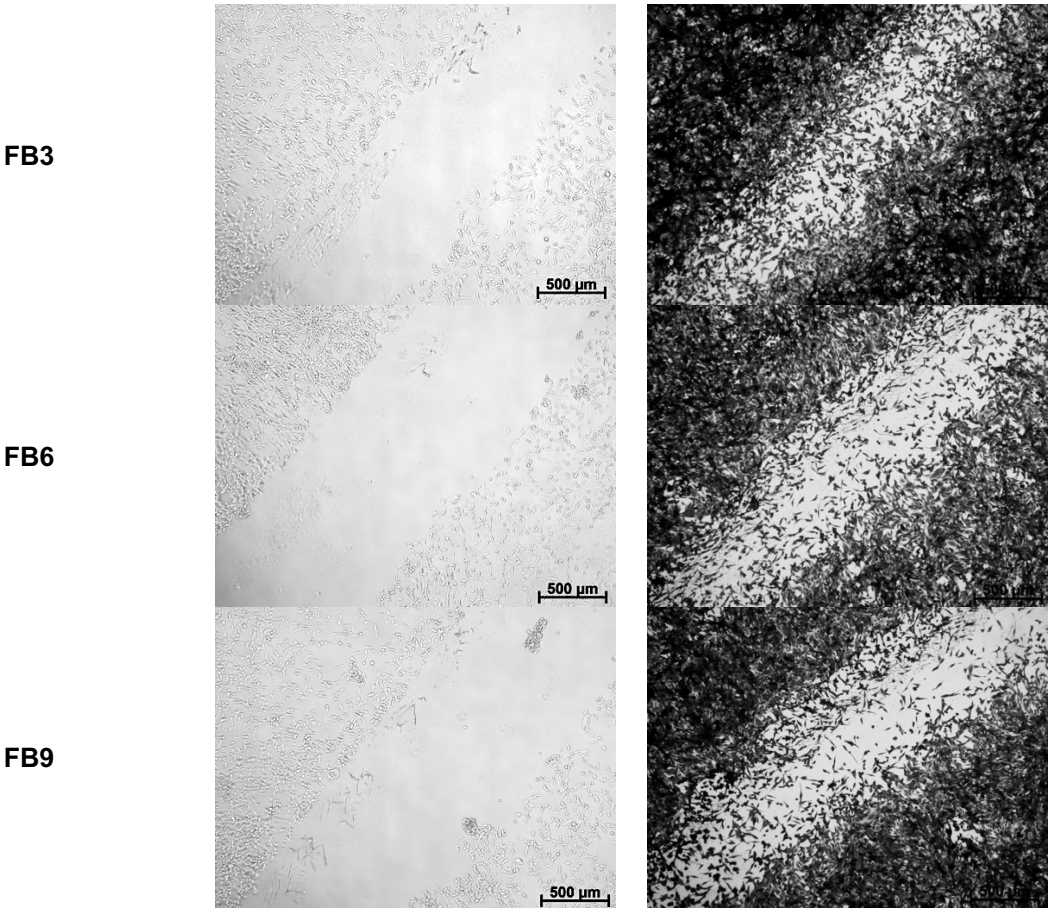
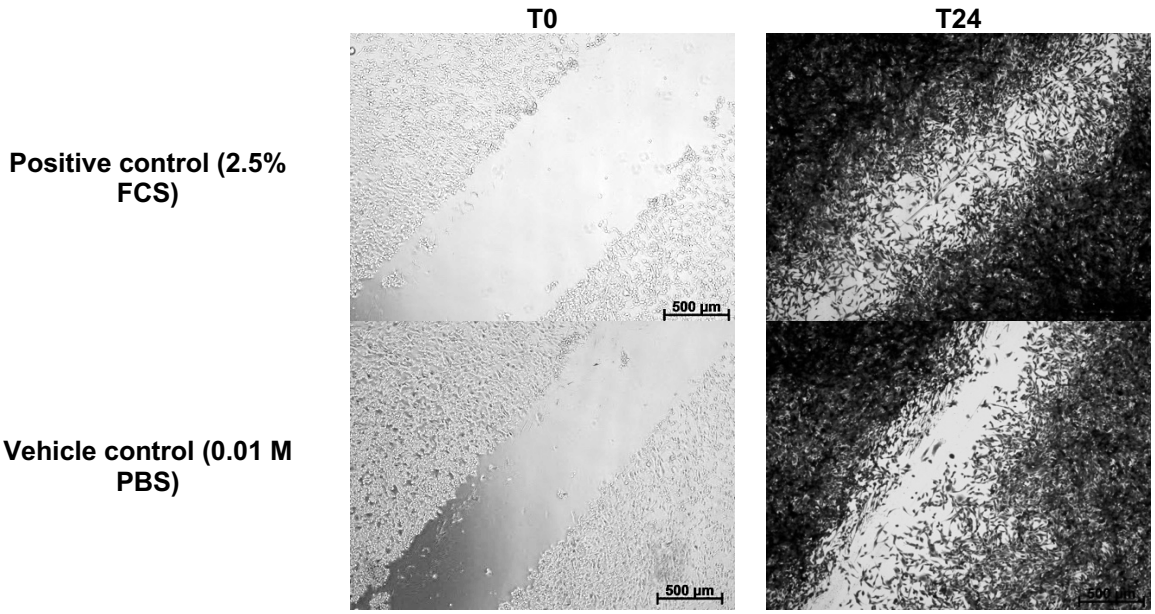
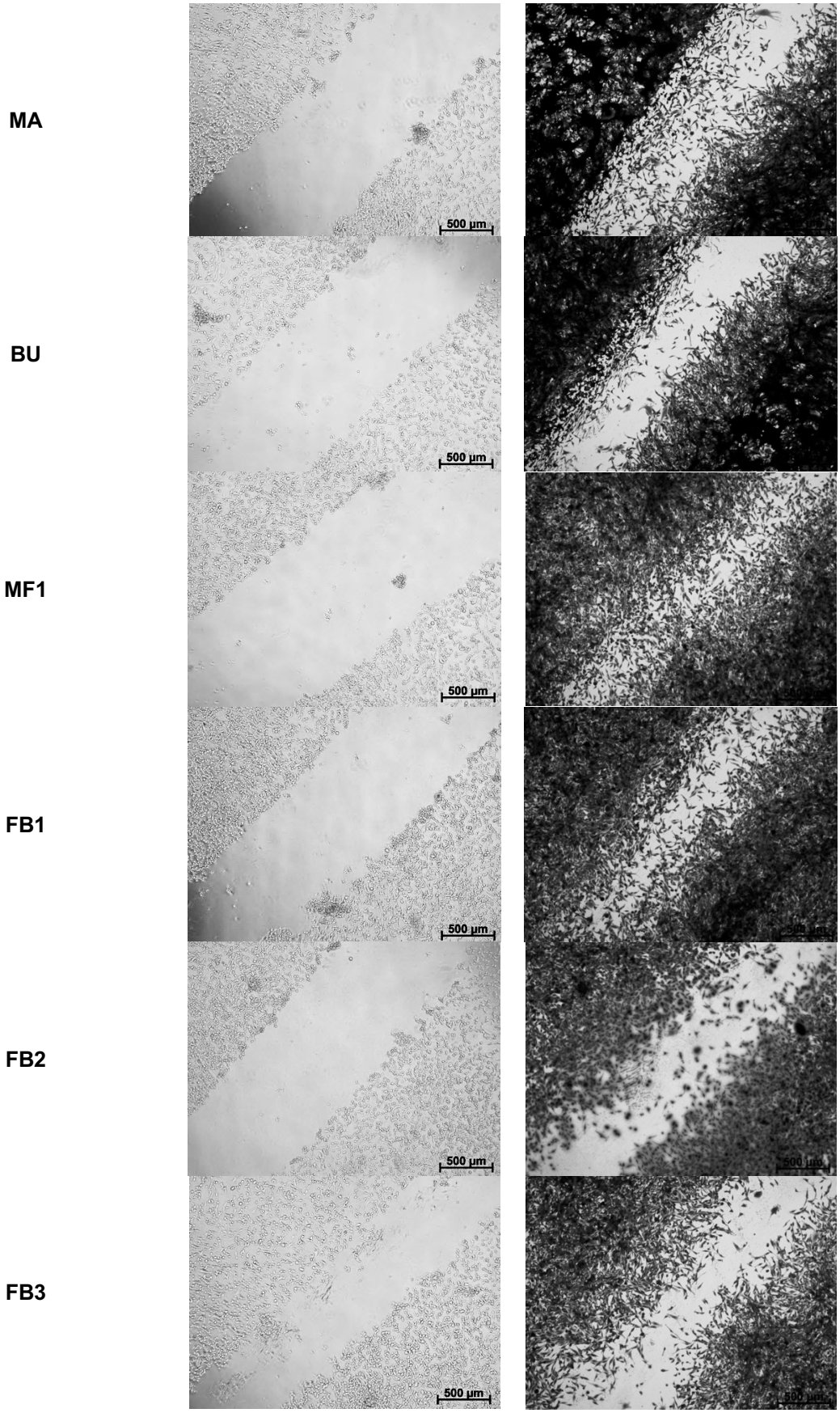


Figure 9.2.4. The cell migration of isolated protein fractions assessed using the scratch migration/HaCaT cell model replicate 1. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.





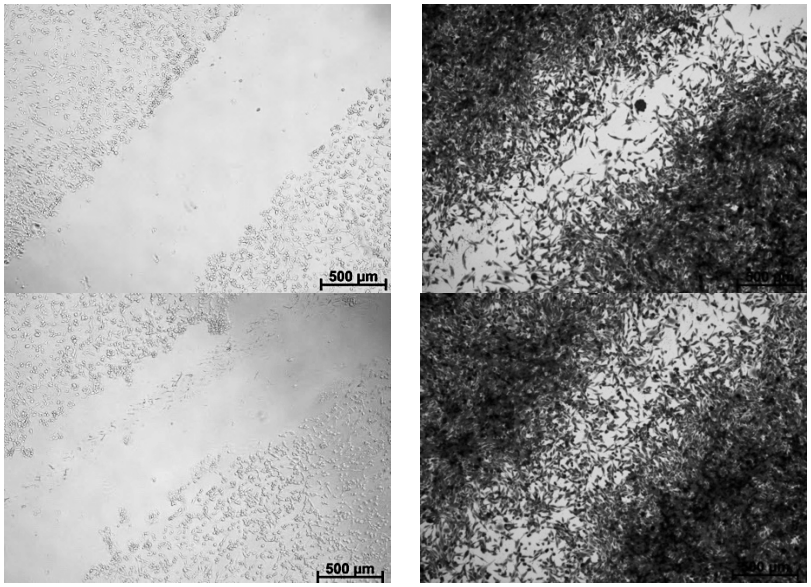
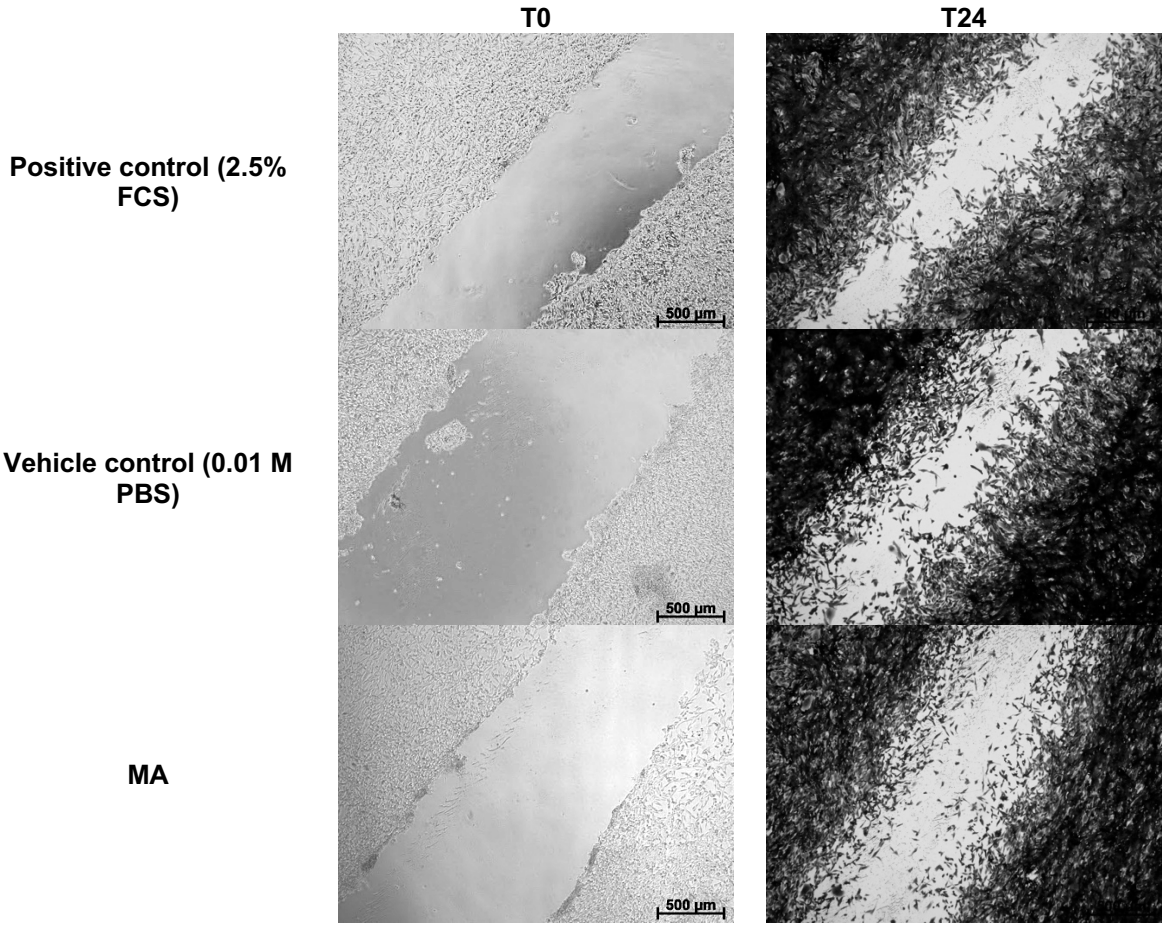
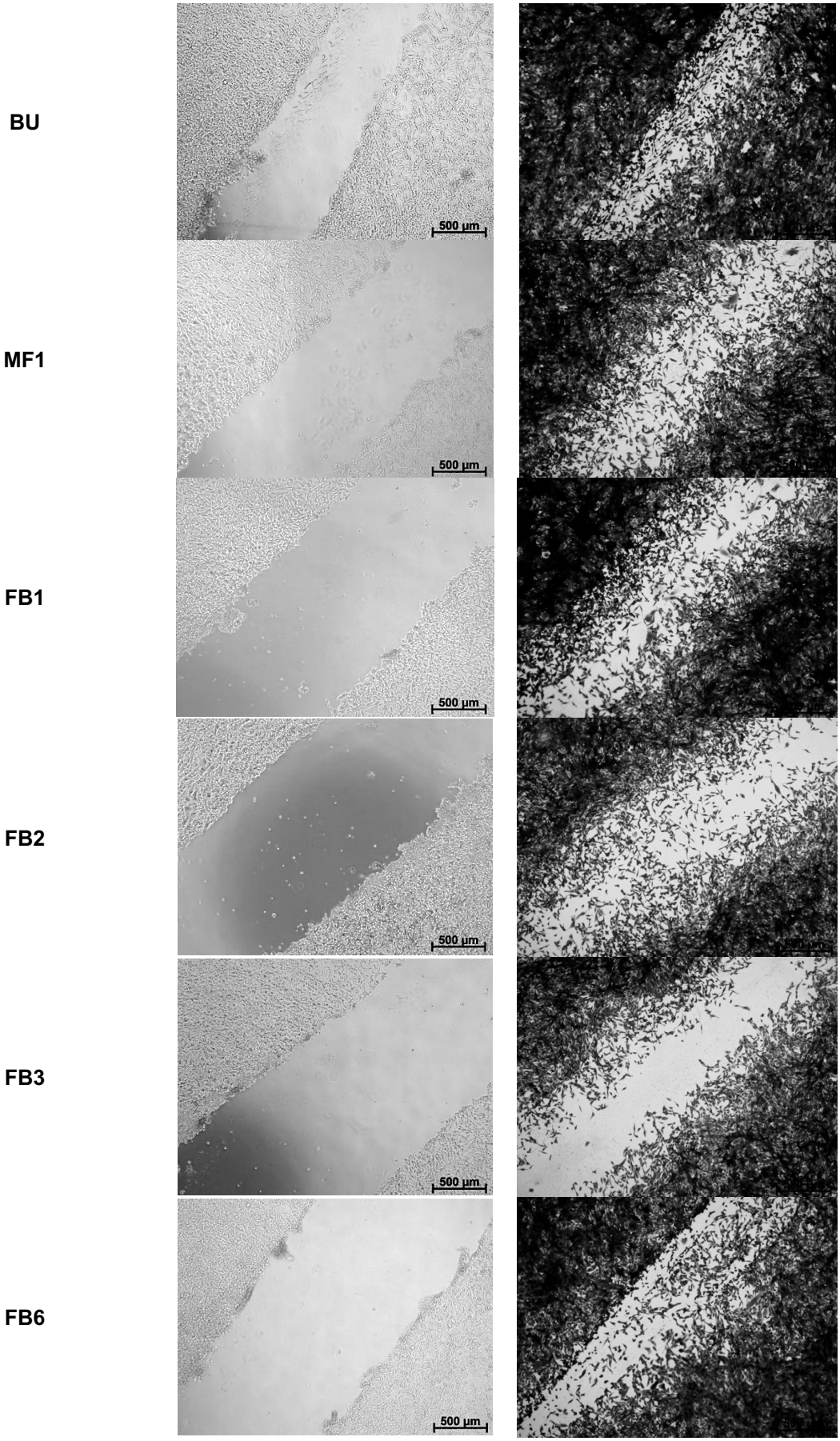


Figure 9.2.5. The cell migration of isolated protein fractions assessed using the scratch migration/HaCaT cell model replicate 2. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.





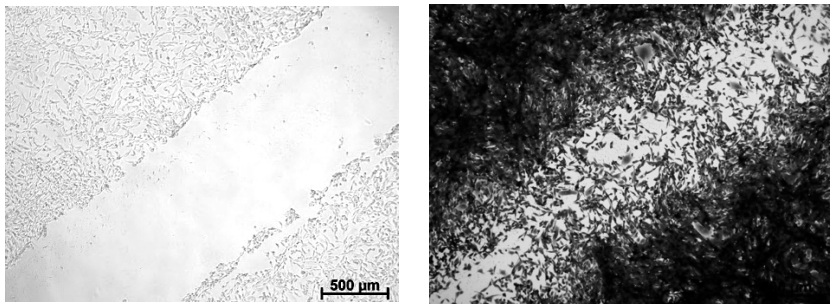
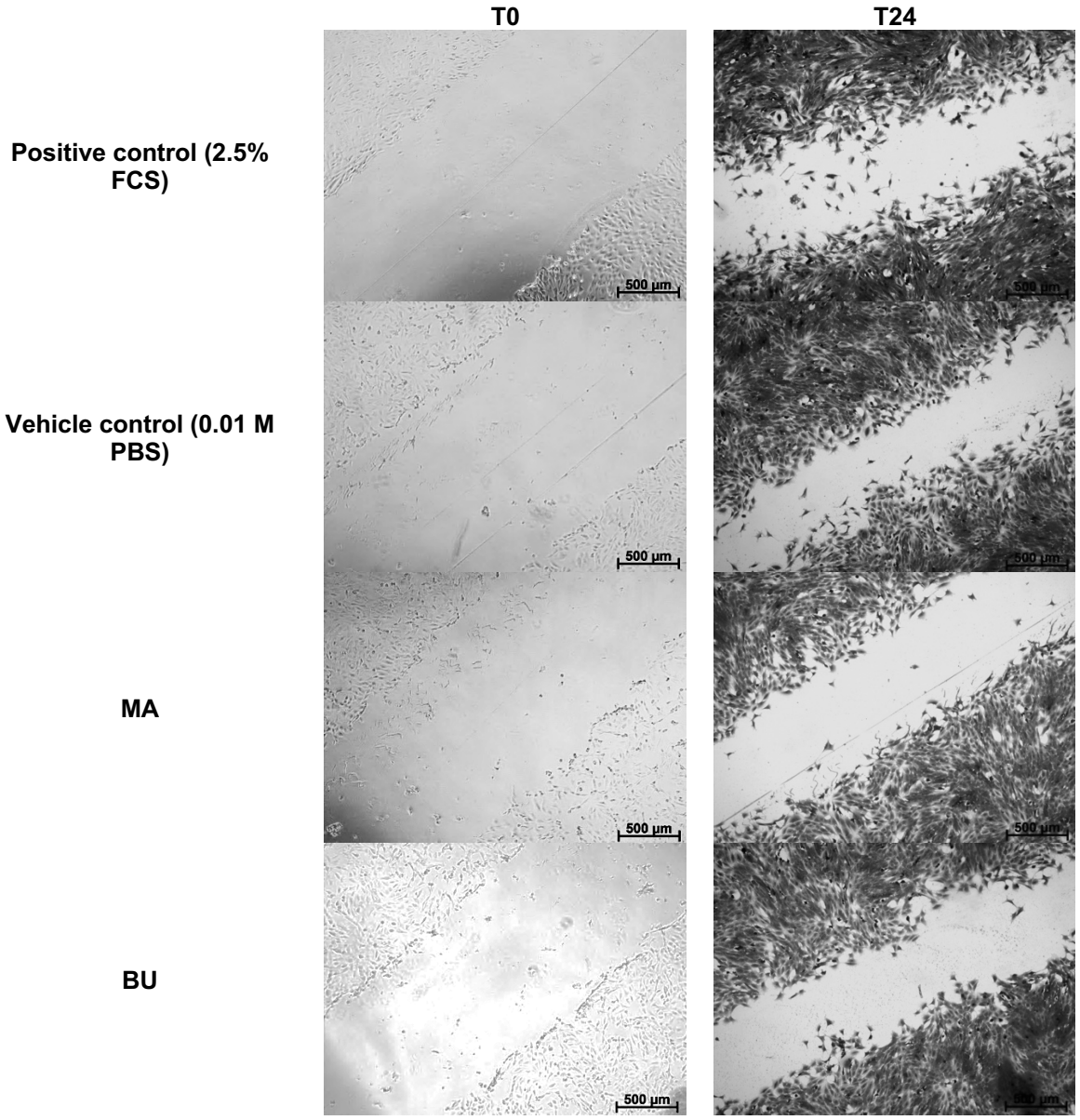
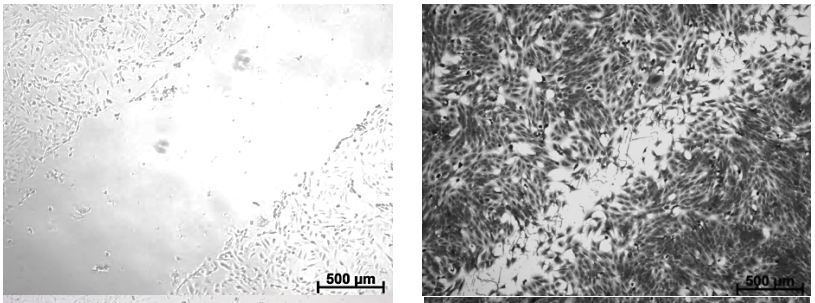


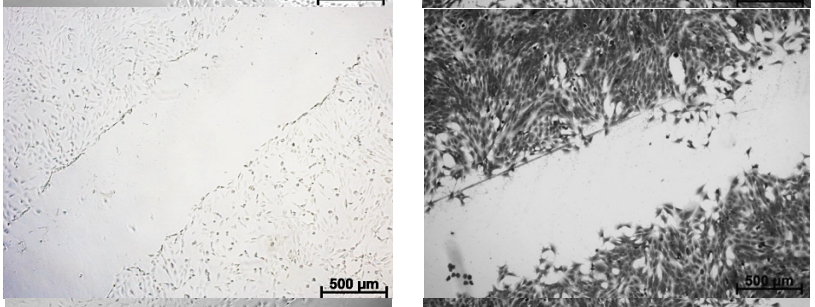
Figure 9.2.6. The cell migration of isolated protein fractions assessed using the scratch migration/HaCaT cell model replicate 3. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.



MF1



FB1



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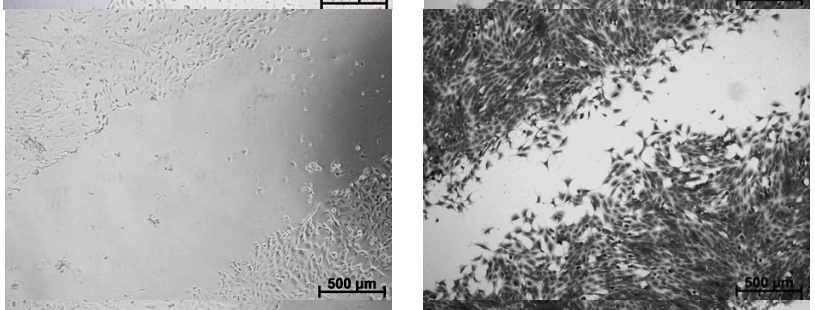

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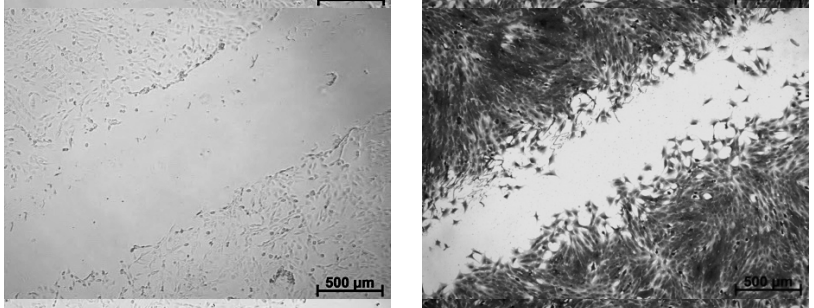
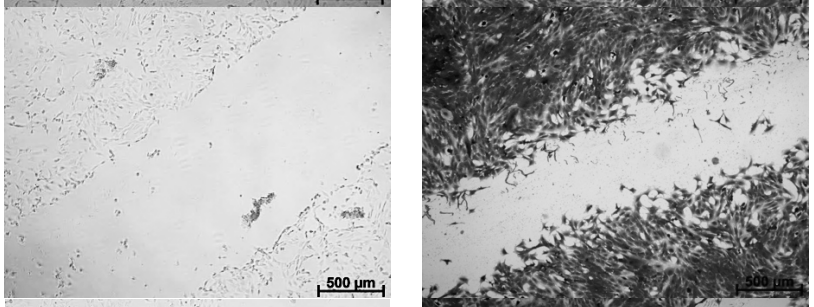


FB6



FB9

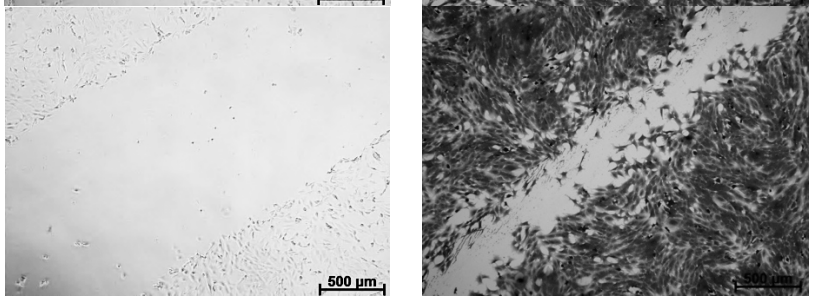
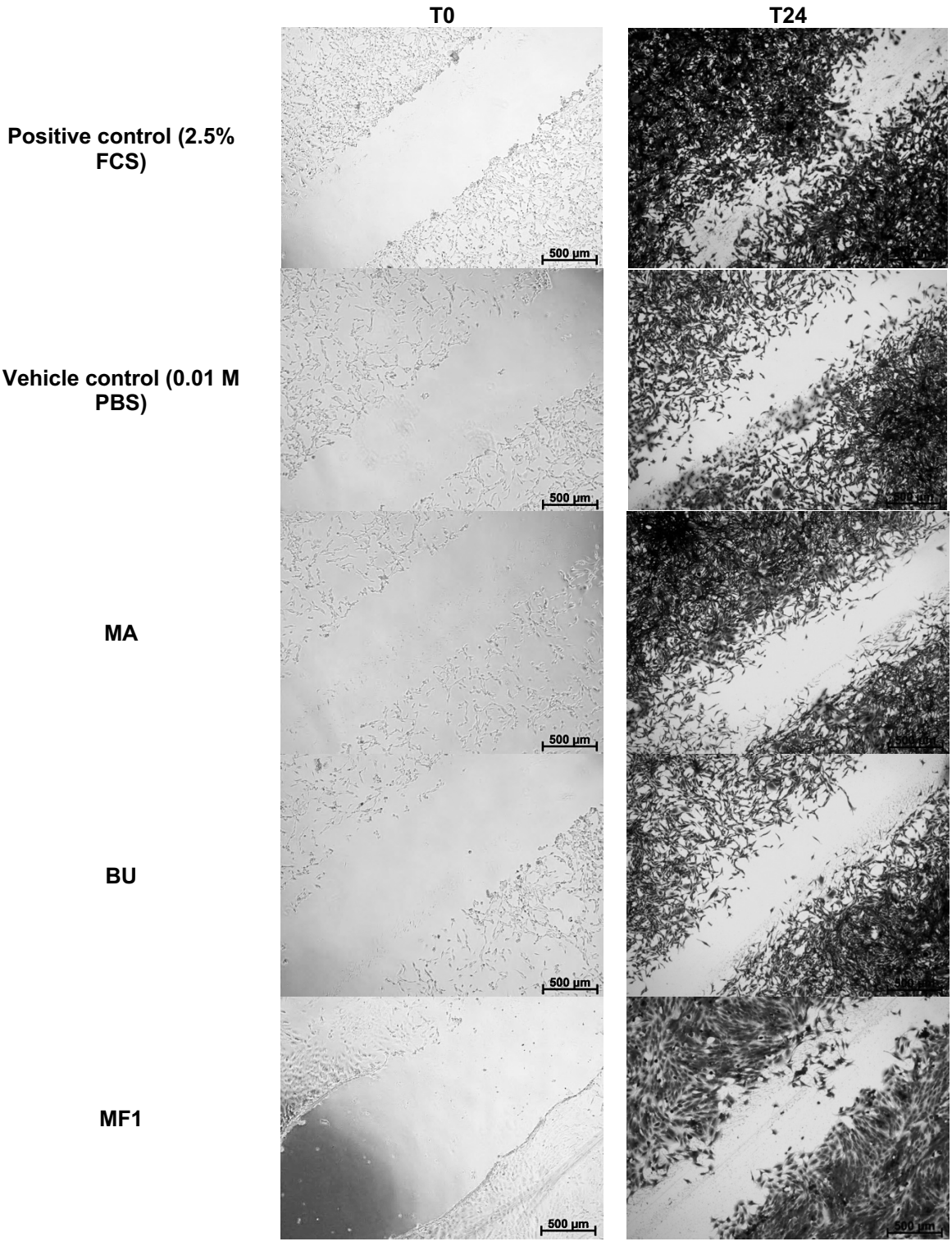


Figure 9.2.7. The cell migration of 0.625% (v/v) whole honey samples assessed using the scratch migration/SC-1 cell model replicate 1. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.



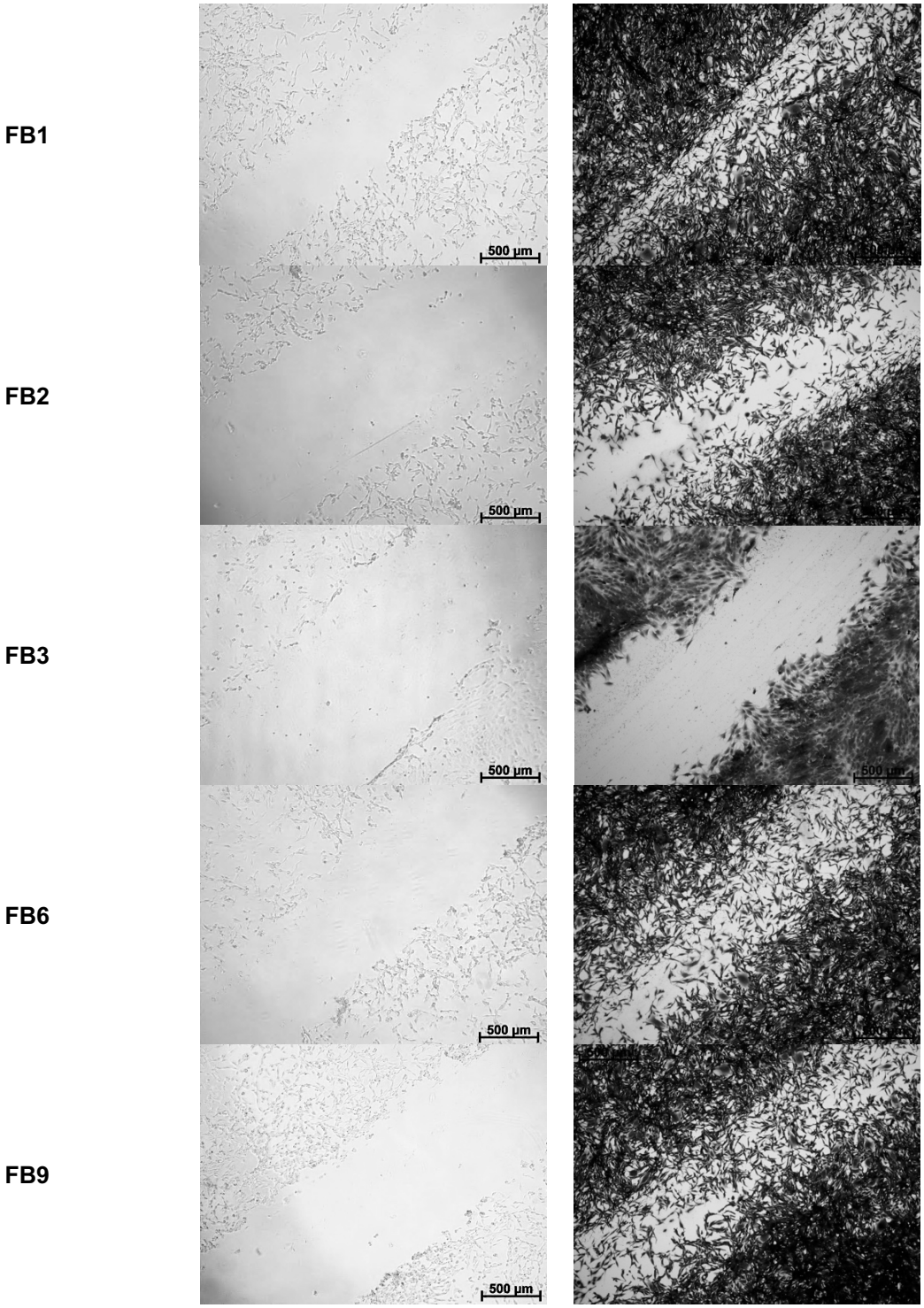
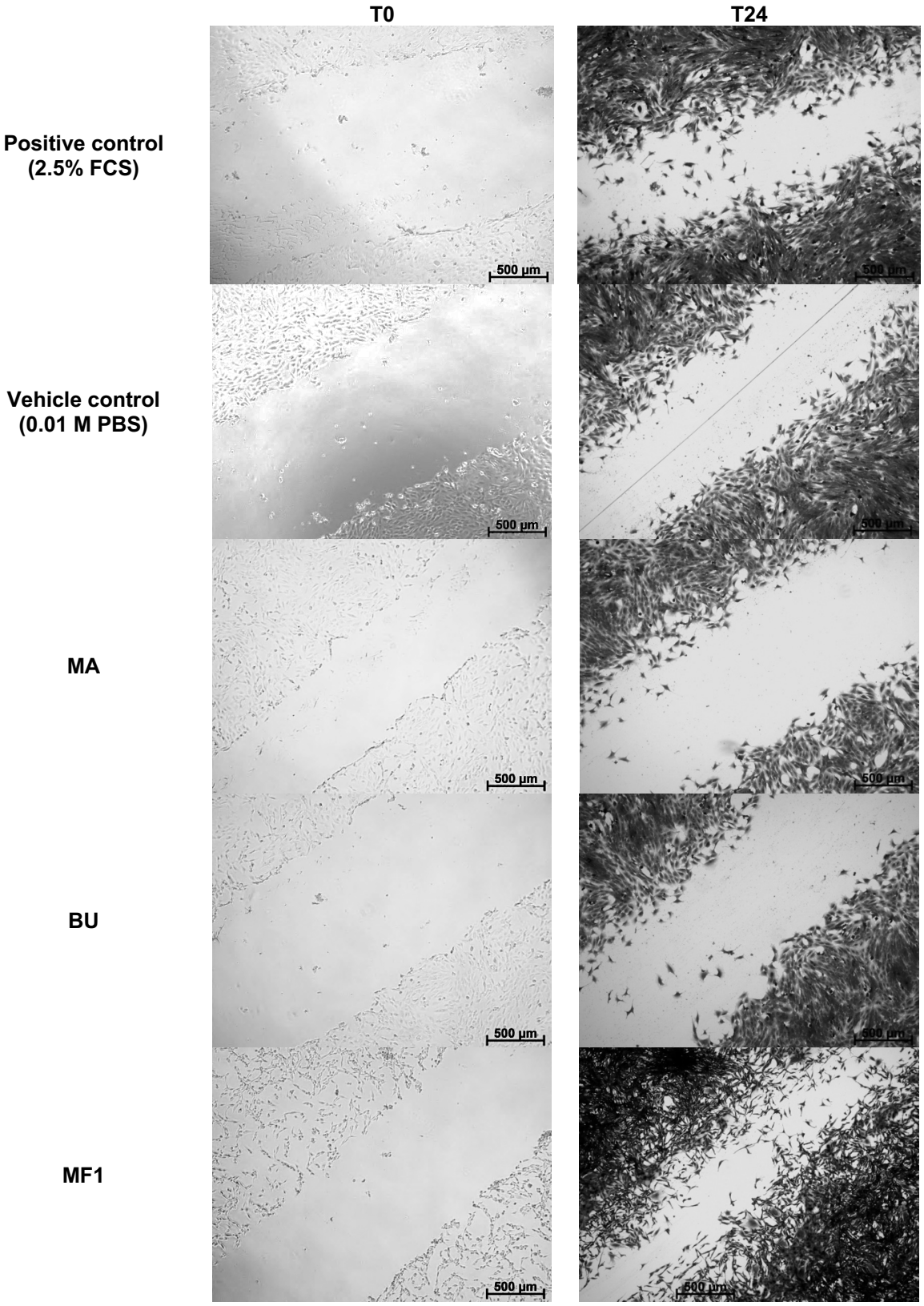


Figure 9.2.8. The cell migration of 0.625% (v/v) whole honey samples assessed using the scratch migration/SC-1 cell model replicate 2. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.



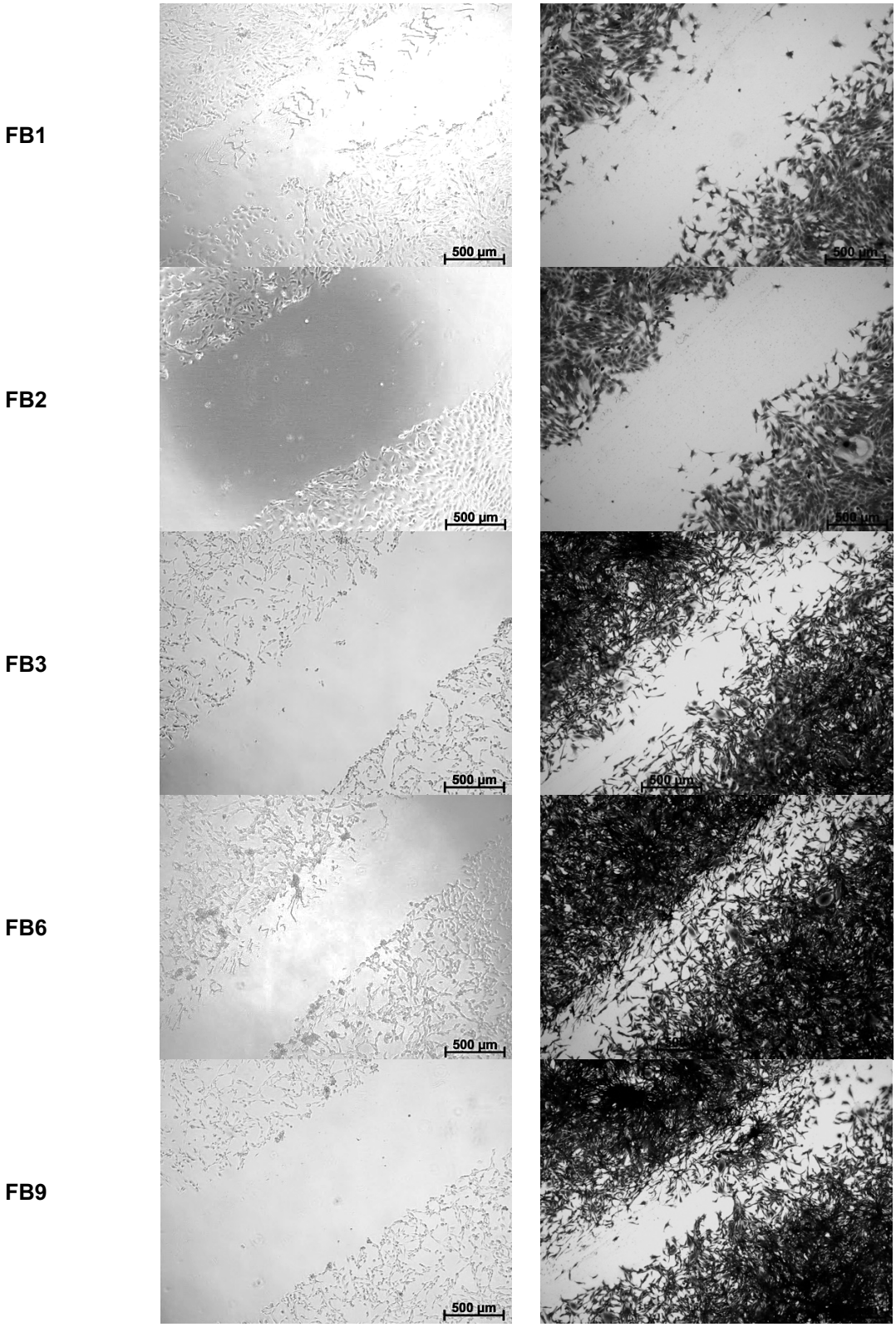
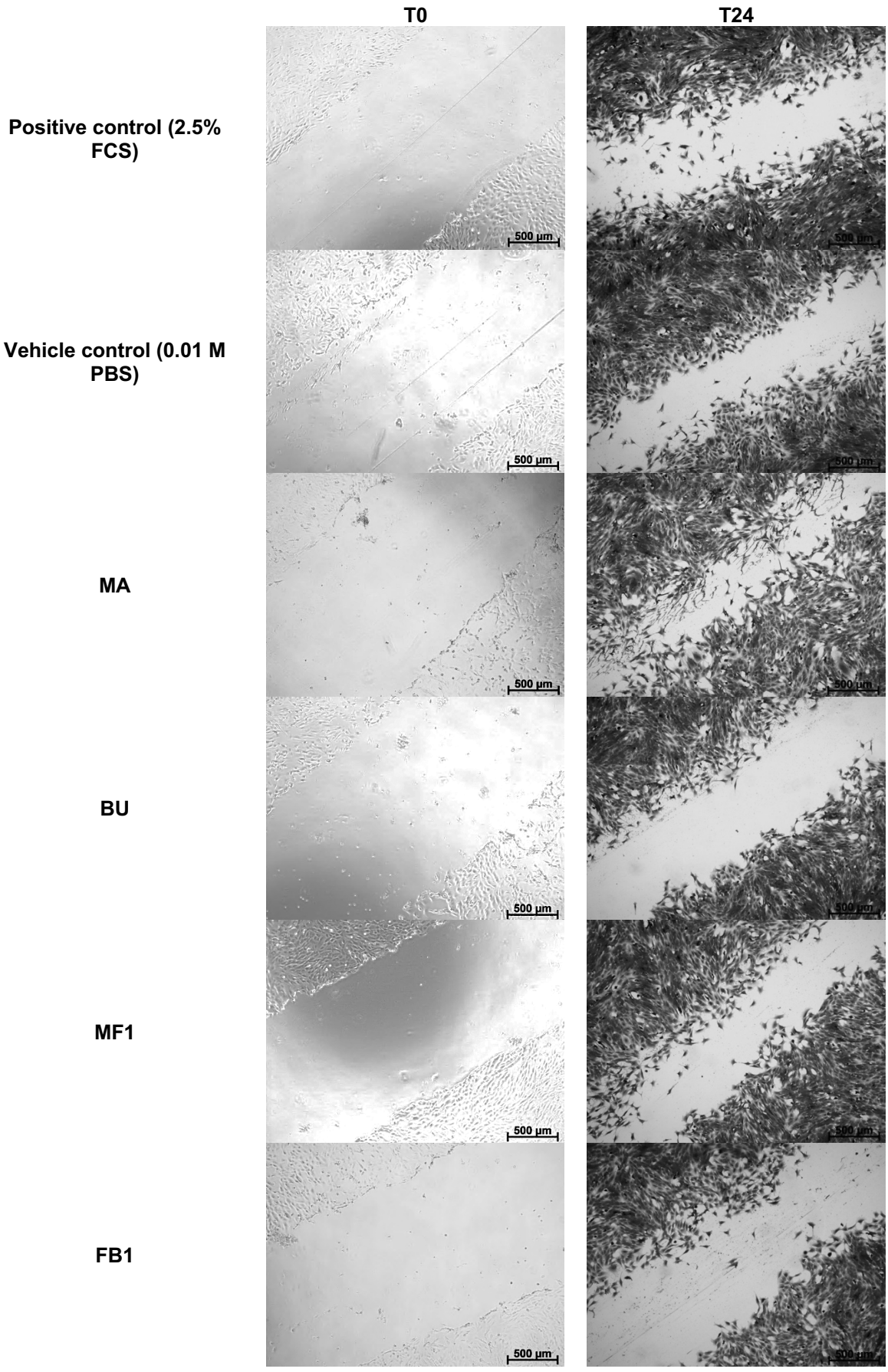


Figure 9.2.9. The cell migration of 0.625% (v/v) whole honey samples assessed using the scratch migration/SC-1 cell model replicate 3. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.



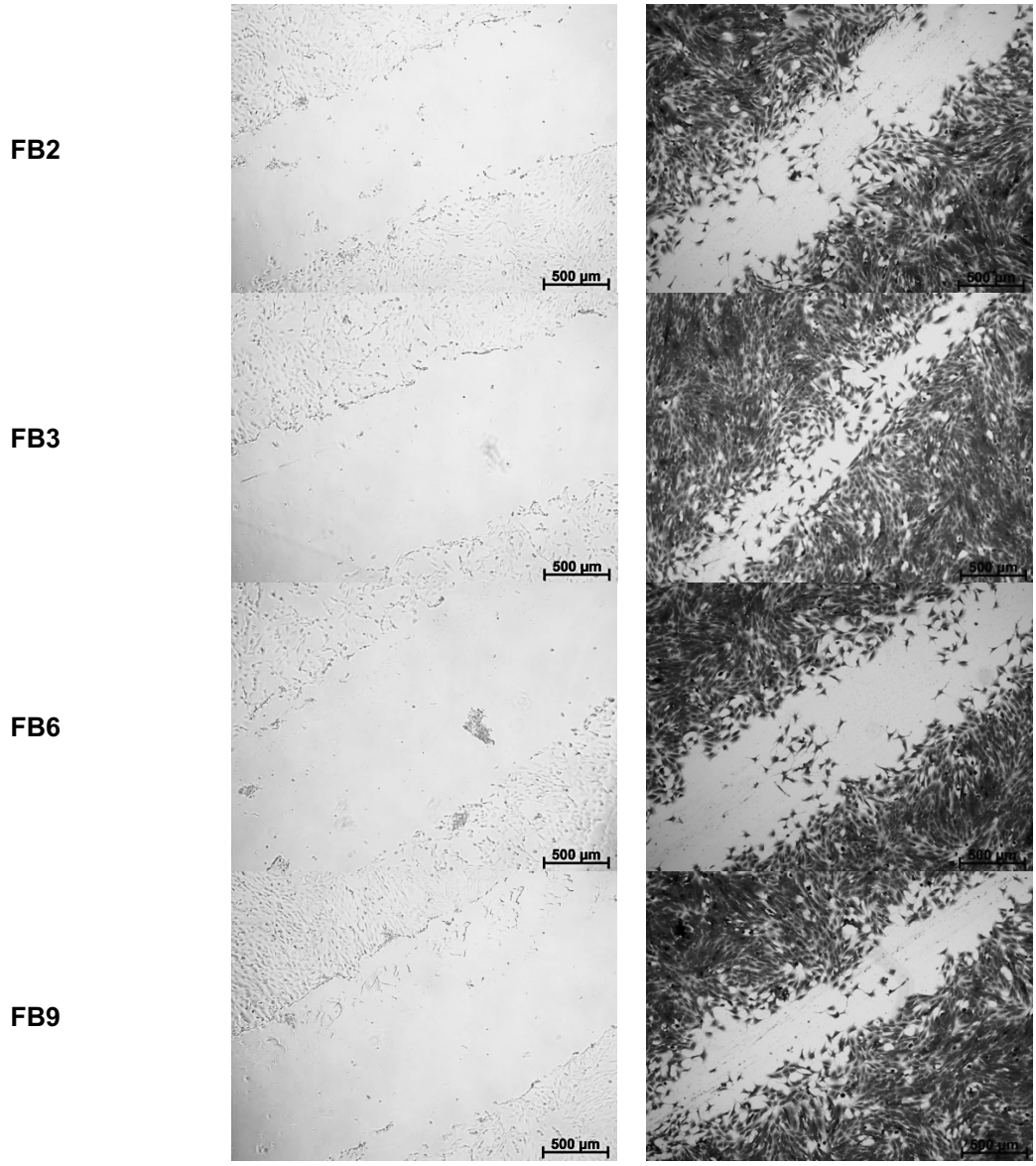
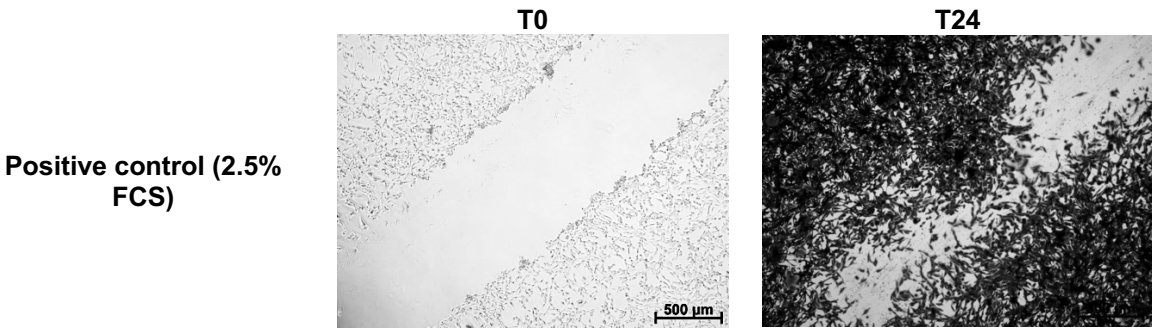
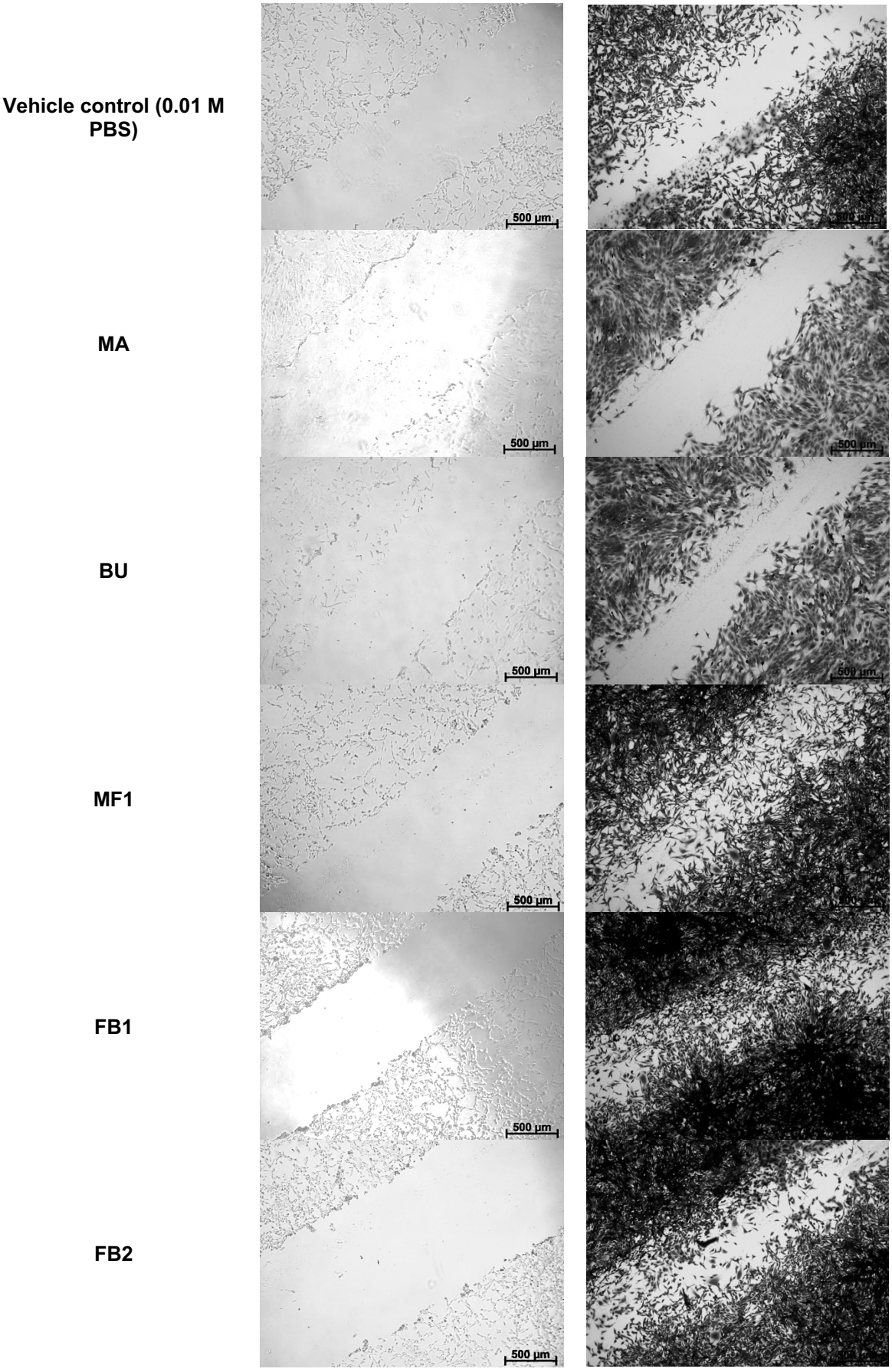


Figure 9.2.10. The cell migration of isolated protein fractions assessed using the scratch migration/SC-1 cell model replicate 1. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.





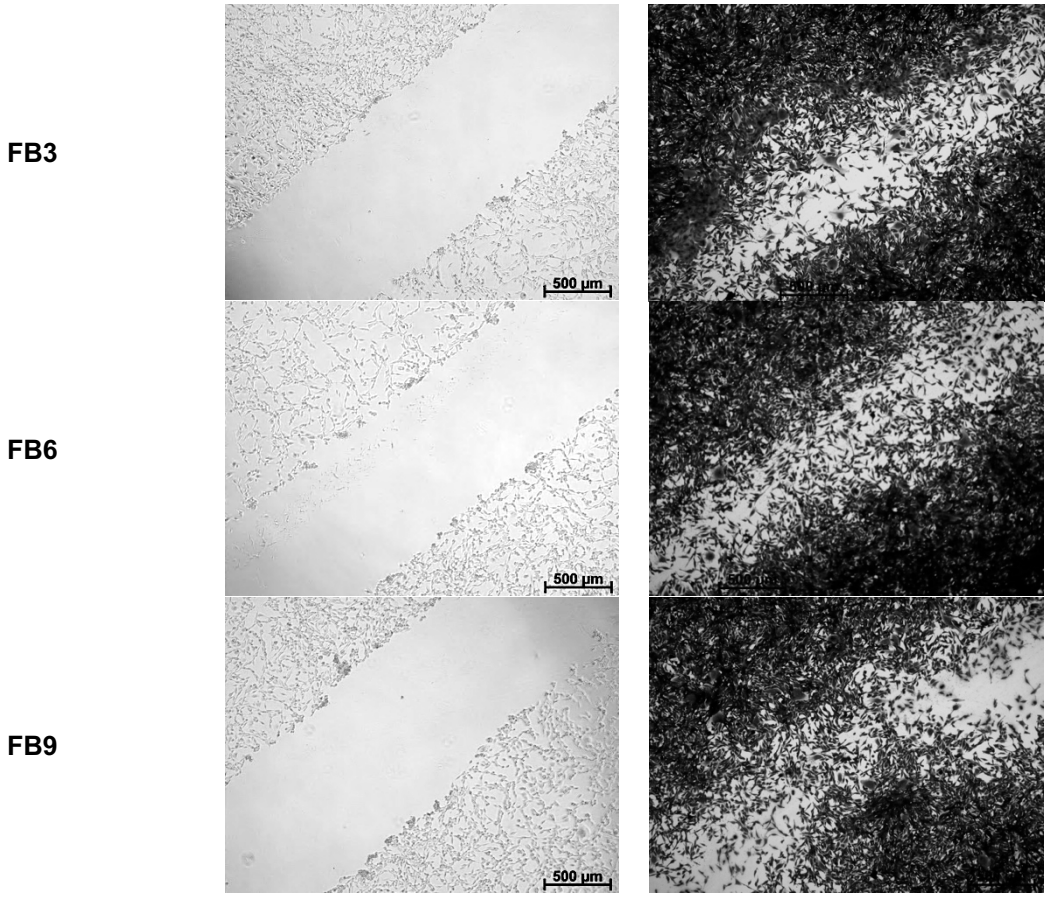
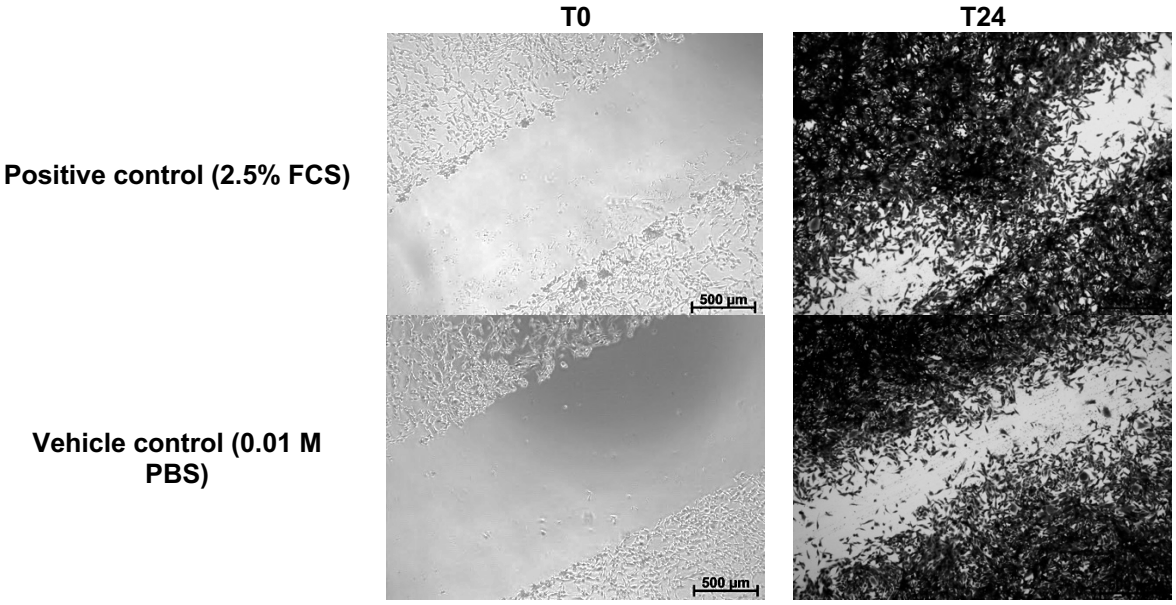


Figure 9.2.11. The cell migration of isolated protein fractions assessed using the scratch migration/SC-1 cell model replicate 2. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.



MA

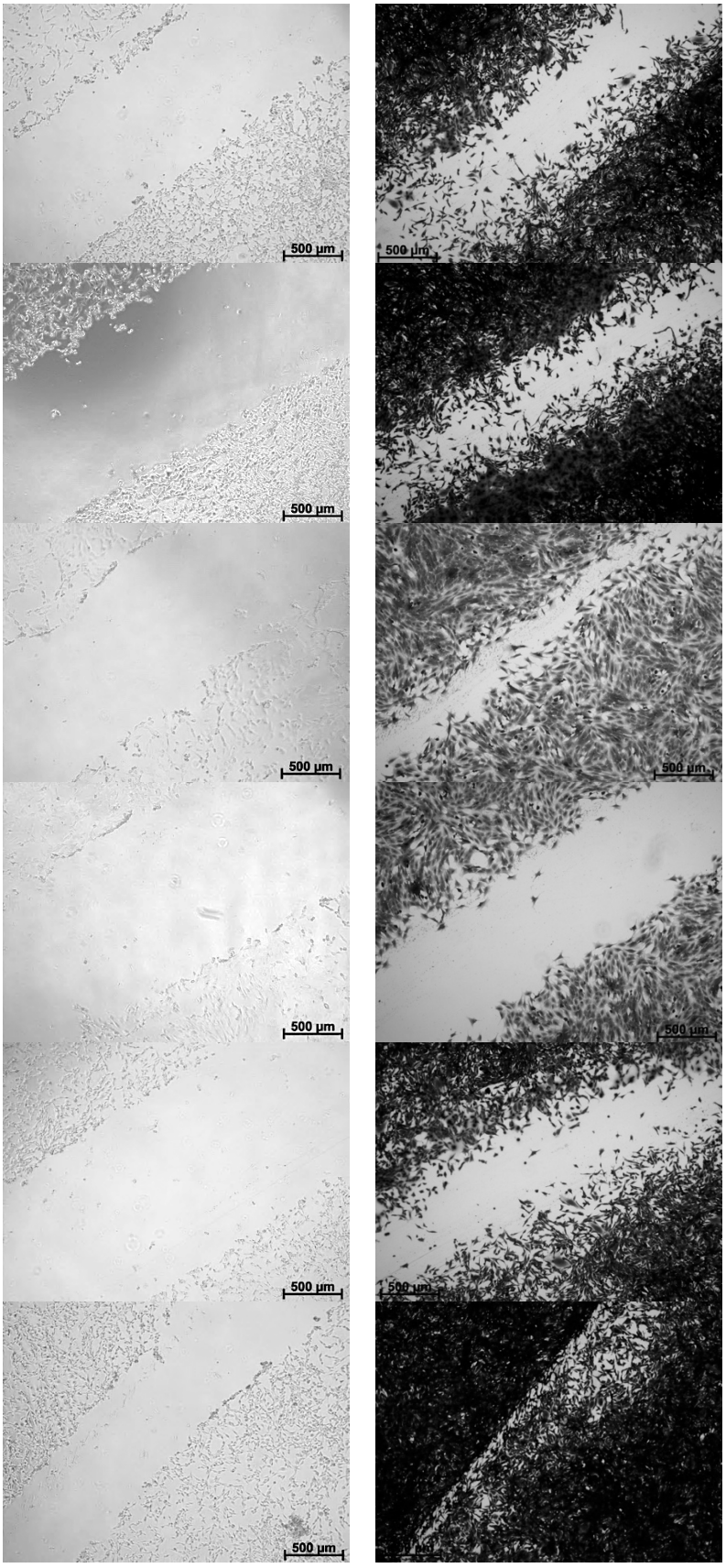
BU

MF1

FB1

FB2

FB3



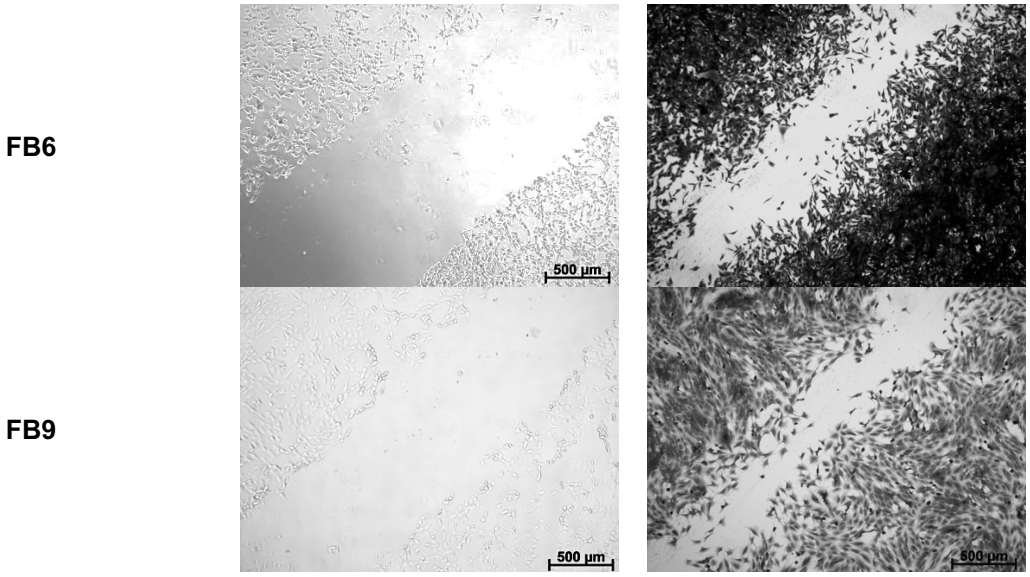


Figure 9.2.12. The cell migration of isolated protein fractions assessed using the scratch migration/SC-1 cell model replicate 3. Images were taken 24 h apart at 4x magnification, following CV staining, indicated as T0 and T24. Scale bar: 500 µM. Images were taken using the Olympus IX71 Microscope, AxioCam ERc5s camera and associated AxioCam vision software. Images were analysed using the ImageJ software.

9.3 Appendix C: Ethics approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through June 30, 2025 and Expires 07/28/2026.

Faculty of Health Sciences **Research Ethics Committee**

23 January 2025

Approval Certificate
Annual Renewal

Dear Miss CJ Stragier,

Ethics Reference No.: 478/2022 – Line 2
Title: The bioactivity of proteins in Southern African fynbos honey

The **Annual Renewal** as supported by documents received between 2025-01-02 and 2025-01-22 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2025-01-22 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2026-01-23.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  a) annual reports;
  b) reports requested *ad hoc* by the REC;
  c) all visitation and audit reports by a regulatory body (e.g. the HPCSA, FDA, SAHPRA) within 10 days of receiving one;
  d) all routine monitoring reports compiled by the Clinical Research Associate or Site Manager within 10 days of receiving one.
- The REC may select your research study for an audit or a site visitation by the REC.
- The REC may require that you make amendments and take corrective actions.
- The REC may suspend or withdraw approval.
- Please remember to use your protocol number (478/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Professor Theresa (TM) Rossouw
***Chairperson**: Faculty of Health Sciences Research Ethics Committee*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).