THE WOUND HEALING EFFECTS OF A 3 CH HOMEOPATHIC SOLUTION OF *HYPERICUM PERFORATUM*, *CALENDULA OFFICINALIS* AND *ECHINACEA PURPUREA* COMBINED WITH PHOTOBIOMODULATION ON DIABETIC FIBROBLASTS

A dissertation submitted to the Faculty of Health Sciences, University of Johannesburg, Johannesburg, as partial fulfilment of the requirements for the degree of Master of Technology: Homeopathy

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Johannesburg, 2019

DECLARATION

I, **Jana Wurz**, declare that this dissertation titled '**The wound healing effects of a 3 cH homeopathic solution of *Hypericum perforatum, Calendula officinalis* and *Echinacaea purpurea* combined with photobiomodulation on diabetic fibroblasts'** is based on my own unaided work, unless where acknowledged, for the Master of Technology in Homeopathy, in the Faculty of Health Sciences, University of Johannesburg, Johannesburg. Neither this work nor any part of this work has been submitted for any degree or diploma for any academic award in any other university.

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ABSTRACT

The skin is the largest organ in the human body and is prone to injury (Martin & Nunan, 2015). Wounds usually heal in a timely, well-regulated fashion by following a predictable sequence of events (Tsourdi *et al.,* 2013). Chronic wounds, however, take longer than three months to heal, and display unique characteristics such as disrupted patterns of cytokine expression and a prolonged inflammatory phase which is detrimental (Martin & Nunan, 2015; Wetzler *et al.,* 2000). Interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) are both cytokines which indicate inflammation (Barrientos *et al*., 2008). Diabetes mellitus (DM) is an endocrine disorder that is reaching epidemic proportion and whose prevalence is increasing worldwide (Cho *et al.,* 2018). In DM, chronic wounds called diabetic foot ulcers (DFUs) are a serious complication which often leads to lower limb amputations (Sohn *et al.,* 2010). The treatment of DFUs is notoriously difficult and new therapies are urgently needed to decrease the burden on national health care systems (Cho *et al.,* 2018).

Homeopathy is a complementary health system which is effective in treating a variety of disease conditions (Fisher, 2012). The substances *Calendula officinalis (C. officinalis), Hypericum perforatum (H. perforatum)* and *Echinacea purpurea (E. purpurea)* effectively stimulates wound healing in herbal form (Parente *et al.,* 2012; Samadi *et al.,* 2010; Rezaie *et al.,* 2013). These remedies are also indicated in the homeopathic literature for the treatment of wounds, however there are limited studies on their effectiveness (Hering, 1997; Boericke, 1999; Sinha, 1981).

Photobiomodulation (PBM) is a therapeutic intervention which involves using low-intensity laser light at specific wavelengths to treat a variety of medical conditions (Andrade *et al.,* 2014). PBM is effective in accelerating wound healing in a variety of experimental models, including DFU in DM (Carvalho *et al*., 2016; Kaviani *et al*., 2011).

The aim of this study was to determine the effects of a 3 cH homeopathic solution containing *C. officinalis*, *H. perforatum* and *E. purpurea* and PBM, both as single therapies and in combination in an *in vitro* diabetic wounded cell model.

Commercially available human skin fibroblasts (WS1) were used as an *in vitro* model in this study. Normal cell models were grown in supplemented culture medium and diabetic cell models were grown in the same with additional glucose to generate a final glucose concentration of 22.6 mMol/L. Cells were sub-cultured at confluency in 3.4 cm diameter culture dishes and subjected to the 'scratch assay' (Felice *et al*., 2015) using a sterile pipette tip to simulate a wound. For the PBM models, cells were irradiated using laser light with a wavelength of 660 nm and a dose of 5 J/cm^2. This dose and wavelength is well established within the research centre, and has been shown to stimulate healing in this *in vitro* diabetic wounded model (Houreld *et al.,* 2018; Ayuk *et al.,* 2016; Jere *et al.,* 2018). For homeopathically treated models a homeopathic solution containing 3 cH *C. officinalis*, *H. perforatum* and *E. purpurea* prepared in 5% ethanol was added to culture medium and incubated for 48 h. In cells treated with both these therapies, the homeopathic solution was added first and then plates were irradiated. Controls were non-wounded cells, and wounded cells that received neither of the treatments, as well as an alcohol control group where 5% ethanol was added to culture medium. Images were captured using a microscope (Olympus CKX41) coupled to a digital camera at 0 h, 24 h and 48 h after treatment to visualise cellular morphology. For biochemical assays, culture medium was collected 48 h post-treatment and cells were detached from culture plates. A proportion of the culture medium was frozen to perform the enzyme-linked immunosorbent assay (ELISA), and a proportion was used immediately to perform the lactate dehydrogenase (LDH) membrane integrity assay to determine cytotoxicity. Detached cells were used to perform the adenosine triphosphate (ATP) luminescence assay and the Trypan Blue exclusion assay, measures of cellular viability. ELISA was performed using commercially available kits to determine the concentration of pro-inflammatory cytokines IL-6 and TNF-α in culture medium.

Morphological changes revealed that both PBM and the homeopathic solution stimulated wound healing in diabetic wounded cells, and that combining these therapies had an even greater effect. Cellular viability was not affected by either homeopathic solution or PBM but combining them caused increased viability as indicated by ATP results. Trypan Blue results revealed decreased viability, contradicting the ATP results. Cytotoxicity was decreased by the homeopathic solution complex and in the combination therapy group. TNF-α concentration remained unchanged with both PBM and homeopathic solution as

single treatment interventions but combining them increased TNF-α concentration. PBM decreased IL-6 concentration, indicating an anti-inflammatory effect.

These cellular changes indicate that combining PBM and a 3 cH solution containing *C. officinalis*, *H. perforatum* and *E. purpurea* holds potential for accelerating wound healing in the context of DFUs. Further research is necessary to establish effects in more complex models.

DEDICATION

To my parents, Sarah Wurz and Carl Wurz, without whom this wouldn't have been possible

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my supervisors. To my co-supervisor: Prof Houreld thank you for your trust, insightful guidance and unfailing support and for being there through every challenge. To my supervisor: Dr Pellow thank you for your encouragement, excellent advice and suggestions and your kindness. Thank you to both of you for giving me the opportunity to complete this work and for helping me reach my goals. Both of you inspired me with your dedication and expertise in your respective fields.

To Sandy, Naresh, Asma, Rahul and the other students and staff at the Laser Research Centre – thank you so much for your help, support and friendly words in and out of the lab. Thanks Sandy for always being there at the critical moments and for your help and encouragement throughout this study.

Thank you to the Laser Research Centre for making this project a reality, for all the cells, reagents and consumables and for the opportunity to work in your lab.

Thank you to all the staff and lecturers at the Department of Homeopathy for the opportunity to study in their department and for all the skills and knowledge they have imparted.

Thank you to Dr Hohl and Dr Manga for their advice and to Fusion Homeopathics for manufacturing the homeopathic solution.

Thank you to the University of Johannesburg for the opportunity to study Homeopathy and for financial support.

Thank you to my friends Ashica, Sam, Celéste, Karabo and Aalia. You always supported me, in so many ways, you guys really are the best.

Last but definitely not least thank you to my parents Sarah and Carl and my grandmother, Alida, for their support, financial and otherwise, and their unconditional love. Thank you for giving me the courage to follow my dreams, I am forever grateful.

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SYMBOLS, ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AGEs	Advanced glycation end-products
C. officinalis	*Calendula officinalis*
cH	Centesimal Hahnemannian potency
COX	Cytochrome c oxidase
cAMP	Cyclic adenosine monophosphate
D/X	Decimal Hahnemannian potency
DM	Diabetes mellitus
DFU/s	Diabetic foot ulcer/s
dH2O	Distilled water
E. angustifolia	*Echinacaea angustifolia*
E. purpurea	*Echinacaea purpurea*
EGF	Epidermal growth factor
p-EGFR	EGF receptor
EtOH	Ethanol
ECM	Extracellular matrix
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
HBSS	Hanks' Balanced Salt Solution
HbA1c	Haemoglobin A1c
HeNe	Helium-neon
h	Hour/s
HIV	Human immunodeficiency virus
HBO	Hyperbaric oxygen
H. perforatum	*Hypericum perforatum*
IL-1	Interleukin-1
IL-6	Interleukin-6
IWGDF	International Working Group of the Diabetic Foot

JAK/STAT	Janus kinase/ Signal transducer and activators of transcription
J/cm^2	Joules per square centimeter
LRC	Laser Research Centre
LED	Light emitting diode
LDH	Lactose dehydrogenase
LLLT	Low-level laser therapy
MCP	Macrophage chemoattractant protein
MEM	Minimum Essential Medium
MIP	Macrophage inflammatory protein
mRNA	Messenger ribonucleic acid
MMPs	Metalloproteinases
mL	Millimetre/s
mmHg	Millimetres of mercury
mW	Milliwatts
min	Minute/s
NEAA	Non-essential amino acids
nm	Nanometer
NIR	Near-infrared
NO	Nitric oxide
NF-κB	Nuclear factor kappa B
n	Number of sample/s
%	Percentage
PVD	Peripheral vascular disease
PBM	Photobiomodulation
PDGF	Platelet-derived growth factor
RCT	Randomised controlled trial
ROS	Reactive oxygen species
rh	Recombinant human
s	Second/s
TIMPs	Tissue inhibitor of metalloproteinases
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha

USD	United States dollar
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

1.1 Foreword

The skin is the largest organ in the human body and forms a barrier between the external and internal environment (Moore, Dalley & Agur, 2010:12). It fulfils many essential functions such as protecting us from harmful microbes and other detrimental environmental conditions (Moore *et al.,* 2010:12). Wounds, or a disruption in normal skin structure and function, commonly occur and activates the process of wound healing, leading to sustained renewal of skin by restoration of anatomy and function (Martin & Nunan, 2015). Wound healing involves a multitude of cell types and chemical messengers such as cytokines, which activates a variety of tightly controlled cellular processes (Tsourdi *et al*., 2013; Diegelmann & Evans, 2004). In chronic wounds the healing process is disrupted and prolonged and characterised by an extended inflammatory phase (Martin & Nunan, 2015). People living with diabetes mellitus (DM) face a host of health complications such as slow-to-heal chronic diabetic foot ulcers (DFUs), which can frequently result in lower extremity amputations (Sohn *et al*., 2010). These wounds are notoriously difficult to treat and living with them negatively affects patients' quality of life (Barg *et al*., 2017; Game *et al*., 2016).

Skin fibroblasts are diverse cells that occur in the dermis (Tracy *et al*., 2016). They play an essential role in wound healing, including breaking down the fibrin clot and generating new extracellular matrix (ECM) (Bainbridge, 2013). They also respond to a variety of cytokines and produce a multitude of them (Barrientos *et al*., 2008). In addition, they are a popular cell model used for *in vitro* studies, especially in the field of wound healing (Felice *et al*., 2015).

Herbal preparation originating from as *Calendula officinalis* L. *(C. officinalis), Hypericum perforatum* L. *(H. perforatum)* and *Echinacaea purpurea* (L.) Moench *(E. purpurea)* have shown wound healing effects (Maver *et al*., 2015; Barnes *et al*., 2001; Rezaie *et al*., 2013). When prepared in homeopathic form (*C. officinalis, H. perforatum and E. purpurea* respectively) these effects have also been demonstrated, but to a lesser extent (Bresler *et al.*, 2007; Raak *et al*., 2012; Pedalino *et al*., 2004). These plants induce wound healing by a variety of mechanisms, such as decreasing inflammation and oedema in the case of

C. officinalis (Muley *et al*., 2009), increasing re-epithelialisation and granulation tissue with *H. perforatum* (Süntar *et al*., 2010; Han *et al*., 2017) and modifying cytokine expression and leucocyte activity in the case of *E. purpurea* (Manayi *et al*., 2015; Oláh *et al*., 2017).

PBM can also be used to stimulate wound healing. This therapy involves using low-powered light at specific wavelengths to modify a variety of physiological processes which results in faster healing (Avci *et al*., 2013). It has been shown to be effective for wound healing in laboratory and clinical trial settings. PBM decreases pain and the wound area and results in faster wound healing (Carvalho *et al*., 2016; Kaviani *et al*., 2011). These affects could be ascribed to increased collagen production and angiogenesis, positive effects on mitochondrial dynamics and altered gene expression of adhesion molecules (Tatmatsu-Rocha *et al*., 2018; Houreld *et al*., 2018).

1.2 Problem Statement

DM is one of the great non-communicable diseases of our time and it was estimated that globally 451 million people between 18 and 99 years old were diabetic in 2017 (Cho *et al*., 2018). DM constitutes a group of metabolic diseases involving persistently elevated glucose levels resulting from defective insulin secretion or action, or a combination of these. Chronic DFUs are a frequent complication of DM, occurring due to a variety of factors present in the disease. Extrinsic factors include trauma or repeated mechanical stress to the feet that have decreased sensitivity due to neuropathy or ischaemia as a result of vascular disease (Tsourdi *et al*., 2013). A thickened basement membrane is central to diabetic vascular disease leading to non-healing ulcers. Intrinsic factors are the direct result of hyperglycaemia on the process of wound healing and could implicate mechanisms such as advanced glycation end-products (AGEs), which lead to the production of inflammatory cytokines as well as abnormal proliferation of keratinocytes. Hyperglycaemia-related effects also lead to decreased fibroblast migration and proliferation (Emanuelli *et al.,* 2016). In addition, decreased immune function in DM could contribute to poor wound healing, possibly due to the abnormal cytokine responses present in DM (Tsourdi *et al*., 2013).

It has been demonstrated that PBM is effective in stimulating wound healing, and also in the context of DFUs (Carvalho *et al*., 2016; Kaviani *et al*., 2011). As a therapy, it has many

advantages such as being safe and convenient, especially with the increasing availability of home-use light emitting diode (LED) devices (Avci *et al*., 2013). The mechanisms driving the healing effects are not fully understood; however, several theories exist including actions on cytochrome c oxidase (COX) and light-sensitive ion channels (De Freitas & Hamblin, 2016).

Basic research on the molecular effects of homeopathic remedies is in its infancy, yet studies reveal that *C. officinalis*, *H. perforatum* and *E. purpurea* can modulate wound healing in homeopathic form. In addition, all three of these plants, when prepared homeopathically,are indicated for wound healing in the traditional homeopathic literature (Hering, 1997; Boericke, 1999).

The unique combination of these homeopathic remedies with PBM has not been investigated.

1.3 Aim of the Study

This study aims to investigate the effects of a 3 cH homeopathic solution containing *C. officinalis*, *H. perforatum* and *E. purpurea* combined with PBM therapy in an *in vitro* diabetic wounded cell model.

1.4 Objectives

The main objectives of this study were to investigate the effect of a homeopathic solution containing *C. officinalis*, *H. perforatum* and *E. purpurea* and PBM using 5 J/cm^2 at 660 nm on:

- the migration and proliferation of normal, wounded, and diabetic wounded fibroblasts,
- the cell viability indicated by the amount of adenosine triphosphate (ATP) released in these models, and by performing the trypan blue assay,
- the cytotoxicity indicated by the amount of lactose dehydrogenase (LDH) released in these models, and
- the expression of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) in these models.

1.5 Hypothesis

Treatment with the homeopathic solution and PBM both singly and combined leads to more effective wound healing compared to controls in diabetic and normal cell models.

1.6 Null Hypothesis

Treatment with the homeopathic solution and PBM both singly and combined does not lead to more effective wound healing compared to controls in diabetic and normal cell models.

CHAPTER 2

LITERATURE REVIEW

2.1 The Skin

2.1.1 Structure and function

The skin forms the integumentary, or covering, system of the body and comprises our largest organ (Moore *et al.,* 2010:12). Skin is composed of three main layers: the epidermis, dermis and subcutis or hypodermis (Figure 2.1) (Young, Lowe, Stevens & Heath, 2006:167). Each layer has a unique structure and is composed of a distinct collection of cells and tissues. Nevertheless, there is a degree of inter-subject variability in the composition of human skin (Groenendaal *et al*., 2010). The epidermis is the outermost layer. It is highly specialised and self-regenerating, and produces the protective, somewhat waterproof protein keratin. It also contains melanocytes which produce pigment for sun protection, Langerhans cells which can recognise foreign antigens, and Merkel cells with touch receptors (Young *et al*., 2006:168). A specialised basement membrane tightly binds the epidermis to the underlying dermis. *Rete ridges* – epidermal downgrowths – penetrate the dermis and provide even stronger tethering (Young *et al*., 2006:168).

The dermis consists of a harder layer with horizontally arranged collagen and elastic fibers with fibroblasts. The dermis, situated beneath the epidermis, is less dense and contains many small blood vessels as well as sensory nerve endings and sense organs in certain areas (Young *et al*., 2006:168). The subcutis is a layer of adipose tissue which is frequently compartmentalised by downward extensions of collagen from the dermis. The function of the subcutis is shock absorption, thermal insulation and fat storage. The dermis and subcutis contain a variety of skin appendages – hair follicles, sebaceous glands, eccrine glands and ducts, as well as apocrine glands in some areas (Young *et al*., 2006:168).

Skin fulfils various crucial functions including containing the body structures and fluids; protecting the organism from potentially harmful environmental conditions such as fluid loss, ultraviolet radiation and microbes; regulating heat by sweating and controlling blood vessel diameter; creating sensations such as pain; and synthesising vitamin D (Moore *et al*., 2010:12).

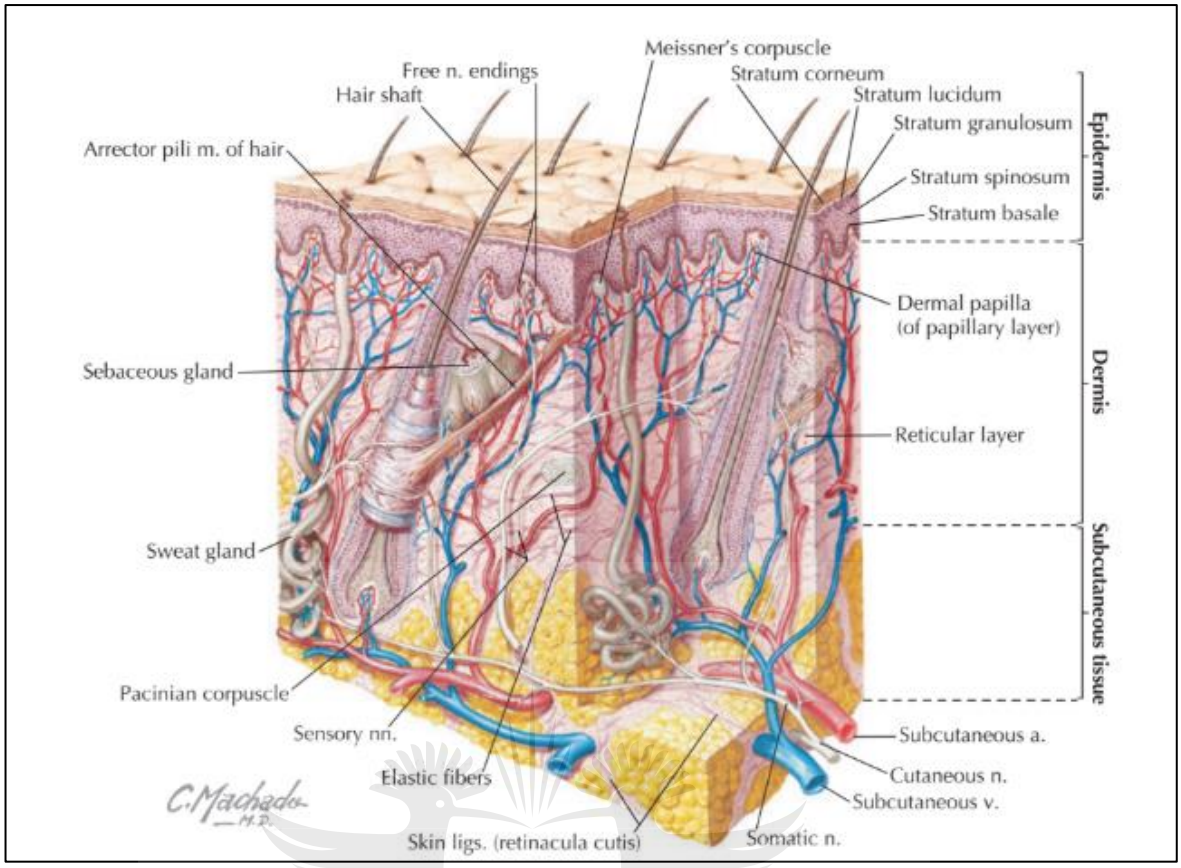

Figure 2.1: Layers of the skin (Hansen, 2017:4).

2.2 Wound Healing

A wound involves damage to the skin and can occur from various processes (Martin & Nunan, 2015). Wounds can be further classified as acute or chronic (Figure 2.2). Acute wounds heal in a highly coordinated fashion which leads to restored skin with its functional components (Martin & Nunan, 2015). Chronic wounds are classified as barrier defects that do not heal within three months and involve a disruption of the typical wound healing process. Chronic wounds (DFUs, venous leg ulcers or pressure ulcers) may often become infected and display an abnormal inflammatory profile (Martin & Nunan, 2015).

Damage to the skin results in activation of wound healing (Martin & Nunan, 2015). Standard wound healing is a complex and dynamic physiological process involving a series of orchestrated events; these comprise the phases of haemostasis, inflammation, proliferation and remodelling, all tightly controlled by various growth factors and cytokines (Diegelmann & Evans, 2004).

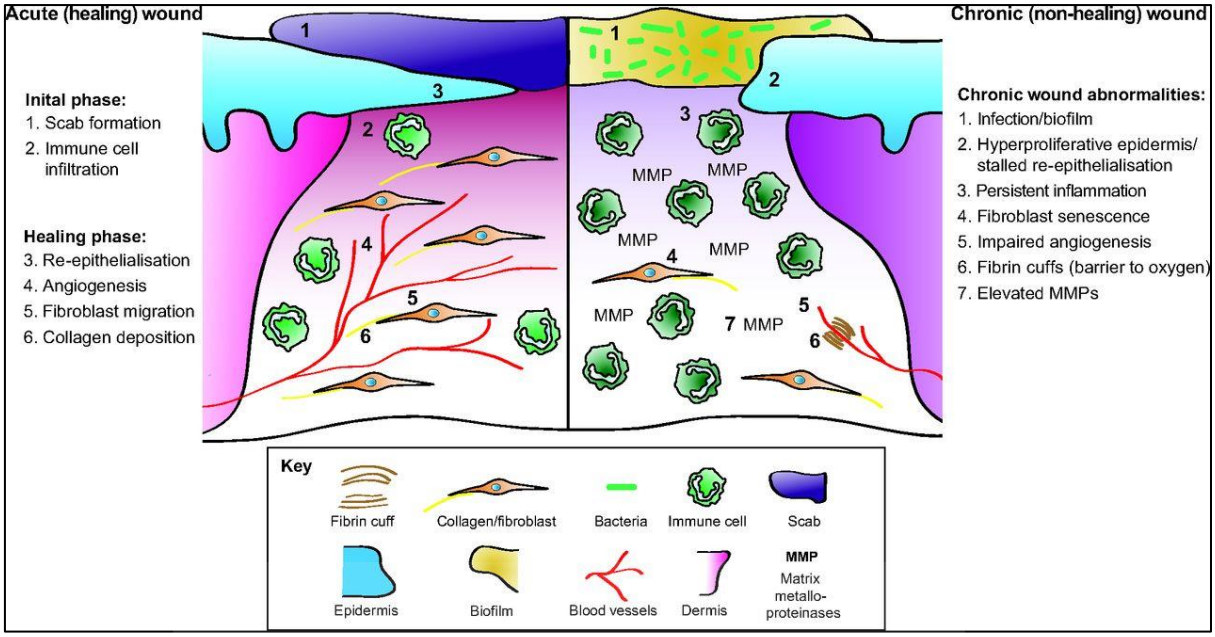

Figure 2.2: The cellular and molecular differences between acute healing wounds and chronic non-healing wounds (Nunan *et al.*, 2014).

2.2.1 Phases of wound healing

The healing process starts at the moment of injury and the first phase, haemostasis, occurs within an hour of injury (Tsourdi *et al*., 2013). Vasoconstriction and clotting are the hallmarks of this phase and both are initiated by platelets. Blood platelets encounter exposed elements of the ECM, such as collagen, which then triggers the release of essential growth factors and cytokines, such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) (Diegelmann & Evans, 2004). These events activate the clotting cascade and initiates the next phase, namely inflammation (Tsourdi *et al*., 2013).

Neutrophils and macrophages play an important role in the inflammatory phase, and this phase lasts up to seven days. Initially, neutrophils enter the wound site and phagocytose debris such as bacteria, foreign materials and damaged tissue (Diegelmann & Evans, 2004). Next, monocytes are attracted to the site where they differentiate into macrophages. They are responsible not only for continued phagocytosis but for releasing a host of growth factors which promote angiogenesis and fibroplasia, which are important initial steps for ECM formation (Tsourdi *et al*., 2013).

The proliferation phase begins two days after injury and can last up to 20 days. Tissue granulation and angiogenesis characterise this phase. Growth factors such as PDGF, macrophage angiogenesis factor and angiotensin are involved with angiogenesis. At the same time, granulation tissue is epithelialised so that it is covered with a barrier of cells (Tsourdi *et al*., 2013). The final phase of wound healing involves widespread remodelling of tissue. This phase lasts longer – from one week to six months post-injury. Proteoglycan and collagen molecules replace the temporary wound matrix and become organised into thicker bundles. In this way scar tissue, stronger but more rigid than skin, is formed (Tsourdi *et al*., 2013).

2.2.2 Cytokines and growth factors involved in wound healing

It is evident that the role of cytokines and growth factors are central to the physiology of wound healing. Successful wound healing depends on the complex integration of these signalling molecules to coordinate cellular functions (Barrientos *et al*., 2008). Growth factors and cytokines are biologically active polypeptides that can change the growth, differentiation and metabolism of their target cells. Binding to specific cell surface receptors or ECM proteins puts a cascade of molecular events into motion, resulting in the transcription of proteins that control various cellular functions (Barrientos *et al*., 2008). The major growth factors involved are the epidermal growth factor (EGF) family, fibroblast growth factor (FGF) family, TGF-β family, PDGF and vascular endothelial growth factor (VEGF).

Some important cytokines involved are interleukin-1 (IL-1), IL-6 and TNF-α. These three cytokines are pro-inflammatory and are increased during the inflammatory phase of wound healing (Barrientos *et al*., 2008). They also all play a role in re-epithelialisation. IL-1 is manufactured by neutrophils, macrophages, monocytes and keratinocytes. It acts on keratinocytes to increase migration and proliferation, as well as activating fibroblasts and increasing FGF-7 release. IL-6 is produced by neutrophils and monocytes and is important for initiation of wound healing (Barrientos *et al.,* 2008). Fibroblasts also release IL-6 and other pro-inflammatory cytokines upon activation of the nuclear factor kappa B (NF-κB) family of receptors (Sundararaj *et al.,* 2009; Flavell *et al*., 2008). Hernández-Caldera *et al.* (2018) demonstrated that human periodontal ligament fibroblasts respond to IL-6 by increasing synthesis of C-reactive protein as well as Th-1 and Th-17-related cytokines – all markers of inflammation. IL-6 stimulates keratinocyte proliferation and

mitosis and attracts neutrophils (Barrientos *et al*., 2008). Its expression is increased 15 to 20 min after injury, with marked expression occurring 60 to 90 min after injury (Grellner, 2002). The importance of IL-6 in wound healing is demonstrated in IL-6 deficient mice. They exhibit delayed wound healing with decreased expression of IL-1, chemokines, adhesion molecules, TGF-β1 and VEGF (Lin *et al*., 2003).

TNF-α can either stimulate or inhibit healing, based on its concentration and on the length of time cells are exposed to it (Barrientos *et al*., 2008). TNF-α expression follows the same timing pattern as IL-6, with peak levels occurring 60 to 90 min after injury (Grellner, 2002). Low levels of TNF-α encourages healing as it stimulates inflammation and the production of macrophage produced growth factors. At higher levels, and especially over prolonged periods, the situation is reversed and TNF-α impedes healing; it suppresses production of ECM proteins and tissue inhibitor of metalloproteinases (TIMPs) while increasing matrix metalloproteinases (MMPs). This situation leads to breakdown of the ECM and collagen, and inhibition of growth factors and target cell receptors, culminating in inhibition of healing. Elevated levels of TNF-α are present in chronic wounds (Barrientos *et al*., 2008).

2.2.3 Fibroblasts and wound healing

Fibroblasts are essential for wound healing, because they fulfil important roles such as breaking down the fibrin clot and generating new ECM and other collagen-rich structures (Bainbridge, 2013). Fibroblasts are the most abundant cell type in the human dermis, yet they are relatively poorly defined. These diverse cells have the remarkable capability to change their function or even transform into new cell types, based on where in the body they are located (Tracy *et al*., 2016). The most common example of this is their ability to transform into myofibroblasts which can contract and is implemented in the process of wound contraction (Tracy *et al*., 2016).

It is still unclear exactly how fibroblasts adapt their structure and function; however, it is thought that both soluble signalling molecules and the cells' extracellular environment play a role (Tracy *et al*., 2016). FGF-2, or basic FGF, promotes fibroblast migration and stimulates their production of collagenase. TGF-β2, TGF-β3 and PDGF also attract fibroblasts to the wound site (Tracy *et al*., 2016). PDGF can increase fibroblast proliferation and thus ECM production, and is responsible for inducing the myofibroblast

phenotype which stimulates contraction of collagen matrices (Barrientos *et al*., 2008). Fibroblasts make at least 17 different growth factors, such as EGF, FGF-2, TGF-β, PDGF and VEGF, which reflects their central role in wound healing (Barrientos *et al*., 2008).

2.3 Diabetes Mellitus

2.3.1. Pathogenesis and epidemiology

DM is one of the great non-communicable diseases of our time and rates have been increasing dramatically over recent decades. It was estimated that globally 451 million people between 18 and 99 years old were diabetic in 2017 (Cho *et al.,* 2018). It is estimated that this figure will rise to 693 million by 2045. Three quarters of people suffering from DM live in low- and middle-income countries. DM significantly increases premature mortality risk and statistics reveal it resulted in 6.8% of global deaths in 2010 (Roglic and Unwin, 2010).

DM constitutes a group of metabolic diseases involving persistently elevated glucose levels resulting from defective insulin secretion, action or a combination of these. It is divided into Type 1 and Type 2 DM, which differ in their pathology and usually in their clinical presentation (Ozougwu *et al*., 2013). Type 1 is characterised by a lack of insulin secretion by pancreatic beta cells, while Type 2 is due to decreased sensitivity of target tissues to insulin – a condition called insulin resistance. Type 1 DM occurs due to an autoimmune reaction which destroys the pancreatic beta cells which are responsible for producing insulin. This condition is most commonly diagnosed in children around four or five years of age, although all age groups can be affected. There is a greater risk for family members of Type 1 DM patients to be diagnosed with it, and to suffer from other autoimmune conditions. The pathophysiology includes deficient insulin production as well as excessive glucagon production, leading to a condition called diabetic ketoacidosis if the patient is not administered insulin. Other derangements include negative energy balance leading to weight loss and increased food intake, increased blood triglycerides and catabolism of proteins (Ozougwu *et al*., 2013).

Type 2 DM occurs much more frequently and accounts for 90% of cases of DM. It is usually diagnosed later in life than Type 1 DM. The prevalence of Type 2 DM has increased significantly in recent years, with poor diet and low physical activity considered to be risk factors for its development (Ozougwu *et al*., 2013). The genetic basis for

developing Type 2 DM has long been recognised, and epigenetic changes play a role in its pathogenesis, providing the link between genetic and environmental factors in this disease. Patients with Type 2 DM have generalised dysregulated DNA methylation, likely with defective protection against oxidative stress and cancer (Karachanak-Yankova *et al*., 2016). Some ethnic groups, such as certain populations of Indians, have a greatly increased risk of developing Type 2 DM (Ozougwu *et al*., 2013). Type 2 DM is characterised by decreased insulin production and increased insulin resistance. Pancreatic beta cell function usually declines gradually. Risk factors that are strong predictors of developing Type 2 DM are a family history of the disease, increased body mass index, increased liver enzymes, obesity, smoking and reduced insulin secretion and action (Lyssenko *et al*., 2008).

Type 2 DM accounts for well over 90% of DM cases in Sub-Saharan Africa (Hall *et al*., 2011). In South Africa the prevalence of type 2 DM is estimated at 9% in people over 30 years (Bertram *et al*., 2013). In addition, significant disability is associated with DM such as retinopathy, amputations, stroke-related disability and ischaemic heart disease-associated disability (Bertram *et al*., 2013).

2.3.2 Chronic wounds in diabetes mellitus

2.3.2.1 Background

Chronic wounds are another complication of DM. Wounds commonly occur on the feet of diabetic patients, and due to a complicated host of factors, they heal poorly and can lead to development of DFUs. DFUs are common, and it is estimated that the lifetime risk of developing a DFU is 25% among the diabetic population (Singh *et al*., 2005). This is a serious complication because a DFU increases the risk for lower extremity amputation (Sohn *et al*., 2010) which in turn increases mortality rates in diabetic patients (Hoffstad *et al*., 2015).

In a systemic review, Rigato *et al*. (2018) found a pooled prevalence of 4.6% for DFUs among diabetic patients in Sub-Saharan countries. DFUs are difficult to treat, and they have a recurrence rate of more than 50% after three years (Boulton *et al*., 2005). This is a great problem especially in Sub-Saharan Africa where complications related to the diabetic foot are a leading cause of hospital admission, amputation and mortality among diabetic patients (Boulton *et al*., 2005). This may be due to unique complicating factors

prevalent in this region such as unhygienic conditions, poverty, co-existing human immunodeficiency virus (HIV) infection, barefoot gait, low income and cultural practices (Boulton *et al*., 2005). These factors have serious implications for the burden on national health systems; for instance the annual cost of treating a diabetic foot was 8 659 US dollars (USD) per patient in Sweden (Ragnarson Tennvall & Apelqvist, 2004), while the estimated global healthcare expenditure on people living with DM was 850 billion USD in 2017 (Cho *et al.,* 2018).

In addition, DFUs affect quality of life. In a qualitative study of patients' experiences, it was found that DFUs and lower extremity amputations were highly disruptive in terms of independence and function (Barg *et al*., 2017). Patients are often immobilised as part of their care and must stop working and suffer a great loss of autonomy during these times (Barg *et al*., 2017).

2.3.2.2 Aetiology of diabetic foot ulcers

Distal sensory neuropathy, autonomic neuropathy and peripheral vascular disease (PVD) occur commonly in diabetics and are the main factors implicated in the development of DFUs (Boulton, 2013). There is no single cause of DFUs, and ulceration usually occurs after the interaction of risk factors (Boulton, 2013). For example, minor injuries often become infected, but the injury remains undetected by the patient due to neuropathy. In this scenario an ischaemic ulcer results due to poor blood supply, caused by PVD.

Peripheral neuropathy is a very important player in the aetiology of DFUs. In sensory neuropathy small sensory nerve fibers that indicate the sensation of pain and temperature are affected (Boulton, 2013). This is a dangerous situation because the foot becomes insensitive to pain, a protective mechanism, leading to a higher risk of injury. Wasting of small muscles in the foot may occur due to the dysfunction of motor nerves. This could lead to foot deformities, resulting from an imbalance between flexor and extensor muscles which causes clawing of the toes and prominence of metatarsal heads; this in turn increases the risk for ulcer formation (Boulton, 2013).

In addition, nerves of the autonomic nervous system are affected in sympathetic autonomic neuropathy. This leads to reduced sweating on the foot, dry skin which may

crack or fissure, and increased blood flow (if the patient does not have PVD) due to impaired vasoconstriction (Boulton, 2013).

2.3.2.3 Molecular mechanisms of poor wound healing in diabetic foot ulcers

Chronic wounds occur due to a combination of factors. These include extrinsic factors and intrinsic factors related to the wound and its biology (Tsourdi *et al*., 2013). Extrinsic factors include trauma or repeated mechanical stress to the feet that have decreased sensitivity due to neuropathy or ischaemia as a result of vascular disease. Vascular disease is a major player in the delayed wound healing of DM. Metabolic abnormalities present in DM produces increased reactive oxygen species (ROS) which causes inflammation and endothelial dysfunction, in this way triggering vascular disease. In addition, the diabetic milieu causes decreased vascular repair mechanisms, altered coagulation pathways and epigenetic activation of pro-inflammatory genes among other effects, further exacerbating the problem (Paneni *et al*., 2013).

One microvascular change is related to the basement membrane of capillaries. A thickened basement membrane has been found in patients with poor glycaemic control (Raskin *et al*., 1983) which leads to decreased elasticity and capacity for vasodilation. This could cause hypoperfusion, which is a mismatch between demand and supply of blood and thereby contributes to delayed healing. Another microvascular change is the 'capillary steal syndrome', a situation where blood is shunted away from nutritive capillaries to subpapillary capillaries due to autonomic neuropathy (Fagrell *et al*., 1999; Chao & Cheing, 2009).

Intrinsic factors related to poor wound healing in diabetics are the direct result of hyperglycaemia itself (Tsourdi *et al*., 2013). Markuson *et al*., (2009) demonstrated the correlation between hyperglycaemia and delayed healing; they showed that individuals with higher haemoglobin A1c (HbA1c) levels, a marker of glucose control, have longer healing times of ulcers. The implicated mechanisms likely involve AGEs. These molecules have a host of deleterious effects such as producing inflammatory cytokines and hindering collagen synthesis (Tsourdi *et al*., 2013).

Decreased immune function in DM, another intrinsic factor, could contribute to poor wound healing, possibly due to an abnormal cytokine response (Tsourdi *et al*., 2013).

Hyperglycaemia increases ROS production which in turn promotes IL-8 release from keratinocytes and neutrophil infiltration (Lan *et al*., 2013). It also inhibits fibroblast migration (Xuan *et al*., 2014). DM is associated with a prolonged inflammatory phase of healing. In a study on genetically diabetic mice, Wetzler *et al*., (2000) demonstrated a sustained inflammatory response with prolonged presence of macrophages and neutrophils, mediated by the chemokine macrophage inflammatory protein (MIP)-2, and macrophage chemoattractant protein (MCP)-1. The pro-inflammatory environment delays healing for various reasons such as reducing wound matrix production and triggering apoptosis and arrest of granulation tissue cells (Acosta *et al*., 2008). In addition, impaired VEGF responses to hypoxic conditions contribute to deficient neovascularization in diabetic tissues (Thangarajah *et al.,* 2009).

Evidence suggests the adverse effects of a hyperglycaemic environment go beyond effects on blood vessels and signalling molecules. Cahn *et al*., (2016) investigated differences in vascularisation and erythrocytes in diabetic patients with and without DFUs. Not surprisingly, they found worse glycaemic control and earlier date of diagnosis in the patients with DFUs. They also demonstrated that erythrocytes in DFU patients had decreased deformability, as well as a larger population of minimally deformable erythrocytes. This could lead to slower capillary flow and thus decreased nutrient supply to injured areas. The mechanism of decreased deformability is suggested to be glycosylation of the proteins present in the lipid bilayer walls of erythrocytes leading to asymmetry in the wall structure. DM also affects mitochondria, leading to mitochondrial dysfunction and disturbed fission-fusion balance (Vincent *et al*., 2010). Hyperglycaemia induces increased fission in neuronal cells, which can have deleterious effects such as disturbed energy production and caspase-3 activation (Vincent *et al*., 2010).

2.3.3 Standard and adjuvant treatment of diabetic foot ulcers

Usually the patient with a DFU is assessed in terms of their vascular status e.g. using pulse assessment, ankle brachial pressure index, Doppler waveform and pulse oximetry, and his or her glycaemic control is optimised (Tsourdi *et al*., 2013). The care of DFUs incorporates standard wound care procedures as well as debridement, moisture dressings, pressure-offloading and infection management (Baltzis *et al*., 2014).

Debridement refers to the process of removing hyperkeratotic epidermis or callus, necrotic tissue, foreign debris and bacteria from the wound bed. This process is necessary to remove these elements that inhibit healing. Moisture dressings are applied to maintain a moist wound environment but also to protect the wound from the external environment, remove wound exudates and encourage regeneration of tissue (Baltzis *et al*., 2014). It remains difficult to develop efficient wound dressings for DFUs. Pressure-offloading, i.e. redistributing load on the plantar surface of the foot, is a vital component in healing DFUs. Surgical interventions such as metatarsal head resection or achilles tendon lengthening can also offload pressure; however, these measures may only add limited additional value to less radical interventions (Baltzis *et al*., 2014). In cases of significant peripheral arterial disease, revascularisation is indicated because blood flow is vital for wound healing and to prevent DFUs and amputation (Baltzis *et al*., 2014). Several revascularising surgical procedures are possible such as angioplasty, endarterectomy, and grafting or bypass, and selection will be based on patient parameters. The last vital component of DFU care is management of wound infection. This involves monitoring the foot for infection, and if present, treating it with oral antibiotics in cases of mild or moderate infection. Patients that have systemic inflammatory characteristics should be investigated for necrotizing infections, gangrene or deep abscesses and may require surgical intervention (Baltzis *et al*., 2014).

New techniques to treat DFUs are continually being developed. Skin substitutes using novel bioprosthetic skin substitutes are an exciting prospect. Usually they consist of a biological substance, such as fibroblasts, in combination with a material to cover the wound (Han & Ceilley, 2017). While experimental results have been positive and encouraging, the costs of this type of treatment remain an obstacle (Han & Ceilley, 2017). There are various specialised wound dressings with beneficial effects such as alginate which is highly absorbent, or honey-impregnated dressings which are anti-microbial and anti-inflammatory (Amin & Doupis, 2016). Several growth factors have been investigated due to their ability to stimulate wound healing processes. A recombinant human (rh)-PDGF dressing is available for use with standard DFU care, and rh-EGF in injectable form had positive effects in neuropathic vs ischaemic ulceration. Ointments containing enzymes such as papain, collagenase or fibrinolysin have been used for enzymatic debridement which promotes tissue granulation. The process of vacuum-assisted closure, where ideally a negative pressure of 125 mmHg is created over the wound area,

is effective in removing wound exudates and reducing oedema (Amin & Doupis, 2016). However, its use is contra-indicated with conditions such as necrosis, ischaemia and where deep tissues (tendons, bones, blood vessels) are exposed. Hyperbaric oxygen (HBO) therapy involves using oxygen at a higher than local atmospheric pressure. It has a host of benefits including reducing tissue hypoxia and oedema and increasing angiogenesis and fibroblast activity. It has been recommended by the European Committee for Hyperbaric Medicine for the management of DFUs. Stem cell therapy is also being investigated and has revealed beneficial effects on angiogenesis (Amin & Doupis, 2016).

The International Working Group of the Diabetic Foot (IWGDF) systematically reviewed the effectiveness of different interventions used to treat chronic diabetic ulcers to guide treatment protocols. The overall picture is that very few interventions, from wound bed preparation, topical applications (including herbal preparations), compression therapy, surgical resection to skin grafts and more experimental treatments such as shockwave therapy are found to be effective for treating DFUs. The problem seems to be a lack of demonstrable effectiveness of these interventions or a lack of robust data due to study design issues. The IWGDF reached the conclusion that there is still no good evidence to justify the use of topical applications or dressing products for DFUs, indicating the great need for new treatments which have undergone rigorous testing (Game *et al*., 2016).

2.4 *In vitro* Models to Assess Wound Healing

2.4.1 The 'scratch assay' and wound healing

Wound healing can be assessed in a variety of ways *in vitro.* One model that is frequently used is the 'scratch assay'. Rodriguez *et al*., (2005) describes it as a 'wound-healing assay', and states that it is a simple and inexpensive method that imitates *in vivo* cell migration during wound healing. The premise of the scratch assay is based on the phenomenon that upon creating an artificial breach (a 'scratch') in the cell monolayer, cells on the edges of the scratch will move towards the gap to close it, eventually restoring cell-to-cell contacts (Liang *et al.,* 2007).

The methodology involves creating a 'wound' in a confluent monolayer of cells. Images are then captured at the beginning, and at regular intervals during cell migration (closure

of the wound) and images are then compared to determine the rate of cell migration (Felice *et al.,* 2015).

Different cell types are used to create the monolayer including fibroblasts, epithelial or endothelial cells. It is most suitable for migration studies but has been combined with other techniques such as gene transfection or microinjection to track individual cells' migration paths on the wound's leading edge (Liang *et al.,* 2007).

There are a number of advantages of using the scratch assay. The first is its simplicity. It is probably the simplest way in which to study cell migration *in vitro*, and it only requires common and inexpensive equipment that is readily available in most cell culture laboratories. Another advantage is the fact that it mimics *in vivo* cell migration of different cell types e.g. fibroblasts will grow as loosely connected populations while endothelial cells will grow as sheets. Lastly, the scratch assay is particularly suited to studying the regulation of cell migration by cell-cell and cell-ECM interactions (Liang *et al.,* 2007).

Disadvantages include the fact that no chemical gradient is established, as in the case of the Boyden chamber assay. It is also more time-consuming than some other methods as one to two days are needed to form a cell monolayer and then 8 to18 h for cells to close the scratch. Finally, it may be resource intensive as large amounts of chemicals and cells are required for the assay, and as such it may not be suited to all studies (Liang *et al.,* 2007).

2.5 Homeopathy

2.5.1 Description

According to the World Health Organization, 'complementary medicine' or 'alternative medicine' refers to health care practices outside a country's conventional or traditional medicine and which are not fully integrated into the main health care system.

Homeopathy is a complementary medicine system founded by Samuel Hahnemann in the late 18th century (Fisher, 2012). It is based on curing disease according to the principle of 'like cures like'. It involves potentising - serially diluting and vigorously mixing/succussing – crude substances to create homeopathic remedies, which are used to treat a variety of disease conditions (Fisher, 2012). It is known as a gentle healing

modality due to its safety profile, and the risks associated with it are modest when compared to conventional medication (Kirby, 2002).

There are many schools of homeopathy, among them 'individualised' or 'classical' homeopathy as well as 'clinical' homeopathy. Individualised homeopathy usually involves the prescription of a single medicine based on the patient's complete array of mental, emotional and physical symptoms, while in clinical homeopathy, one or more homeopathic medicines are prescribed based on certain clinical diagnoses. Clinical homeopathy focuses more on physical symptoms than mental symptoms and more often involves the use of lower potencies, i.e. less dilute medicines such as the 3 cH, 5 cH and 9 cH potencies (Fisher, 2012).

'Potency' refers to the specific dilution of a homeopathic medicine that can range from raw plant extracts called 'mother tinctures' to ultra-diluted LM potencies (Jütte & Riley, 2005). The distinction between 'high' and 'low' potencies is not clearly defined; however a practical boundary can be seen as the 12 cH point or 24 X/D (a dilution of 10^{-24}) which is the same dilution but expressed in decimal potency (X or D) instead of centesimal (C or cH) potency (Jütte & Riley, 2005).

Homeopathy should not be confused with herbalism. Despite the fact that many of the crude materials used to make homeopathic medicines are plants or herbs, they are used in a highly diluted form in homeopathy and are prepared and prescribed differently to herbal medicines (Fisher, 2012).

2.5.2 Homeopathic mother tinctures versus herbal tinctures and extracts

In terms of clinical indications, there may often be no distinction between the use of herbal tinctures or extracts and homeopathic mother tinctures, especially as there is a large amount of overlap between traditional uses of substances. The main difference is in the rationale used to prescribe – whether the prescription is made according to the law of similars (homeopathic use) or according to phytotherapeutic literature (phytotherapeutic use) (Jütte & Riley, 2005).

Herbal substances come in many forms and are produced using different manufacturing processes (Vlietinck *et al*., 2009). Herbal preparations can be primarily classified as

powdered/comminuted, herbal extracts, or dry extracts. Herbal extracts can then be further identified as liquid extracts/tinctures, semi-solid extracts or dry extracts. The difference between a liquid extract and herbal tincture is the ratio of herbal substance to solvent used for extraction. Liquid extracts are made by using 1 part of a plant with 1 to 2 parts solvent, while tinctures are made using 1 part of a plant with 5 or 10 parts solvent (Vlietinck *et al*., 2009).

It can be difficult to distinguish homeopathy from herbalism/phytotherapy when considering herbal extracts and homeopathic mother tinctures, because both are essentially liquid extractions made from plants in most cases. Mother tinctures are a mixture of plant juices and alcohol of a specified concentration, extracts of plants/plant parts or animal parts or secretions mixed with the specified liquid vehicle or nosodes (preparations derived from disease processes) (German Homoeopathic Pharmacopoeia, 2012:H5.1). They are prepared according to manufacturing procedures stipulated in specific homeopathic pharmacopoeias (see Appendix A for manufacturing methods), which are different to the manufacturing methods used to make herbal preparations (Jütte & Riley, 2005).

2.5.3 *Calendula officinalis* L. *(C. officinalis)*

C. officinalis, or the pot marigold, has been widely studied and has a reputation for promoting wound-healing (Maver *et al*., 2015). The phytoconstituents found in this medicinal plant include triterpene glycosides, fatty acids, alcohols and flavonoids (Arora *et al*., 2013). *C. officinalis* has anti-pyretic, anti-tumour, cicatrising, anti-fungal and anti-septic effects. It is used worldwide in various complementary medicine systems mainly in the treatment of skin conditions such as burns, cuts, rashes, varicosis, and ulcers, where it is usually prepared and applied in the form of an infusion, tincture or ointment (Arora *et al*., 2013).

Calendula flower preparations are used in phytotherapy to treat wounds and burns, gastro-intestinal inflammatory disorders (in combination with other herbs), gingivitis and nappy rash (Braun & Cohen, 2010:283-284). It is revered for its non-irritant, antiseptic and healing properties and can be used both internally and externally (Braun & Cohen, 2010:284).

Its anti-inflammatory effects are attributed to oleanane-type triterpene glycosides and triterpenoids, while its anti-oedema effects are due to faradiol esters (Muley *et al*., 2009). Wound healing effects, indicated by markers such as collagen-hydroxyproline and hexosamine and levels of acute phase proteins, may be due to decreased lipid peroxidation secondary to the antioxidant effect of *C. officinalis* (Muley *et al*., 2009).

2.5.3.1 Wound healing effects of *C. officinalis*

Several research studies have been conducted to investigate the wound healing effects of *C. officinalis* and these illustrate its great healing potential. Fronza *et al*., (2009) studied the effect of hexane and ethanolic extracts of *C. officinalis* on wound healing by using mice fibroblasts with the scratch assay and then evaluating wound closure by fluorescent microscopy. It was found that *C. officinalis* herbal extracts stimulated fibroblast proliferation and migration. Parente *et al*., (2012) used rats and a chorioallantoic membrane model to investigate the wound healing properties of an ethanolic extract of *C. officinalis* applied topically. They found that it had anti-inflammatory, antibacterial, angiogenic and fibroplastic properties. Tanideh *et al*. (2013) induced oral wounds in hamsters and tested the efficacy of a 5% and 10% *C. officinalis* gel application, which was applied once daily for five days. Animals treated with *C. officinalis* healed faster than control groups based on microscopic and macroscopic wound evaluation.

Not much research has been completed on *C. officinalis* in homeopathic dilutions or potency, however existing results indicate a potential for increasing wound healing. *C. officinalis* is indicated in homeopathic texts for ulcers of the lower limb with pain and suppuration and for preventing necrosis (Hering, 1997:258) and Boericke (1999:156) states that it ‘promotes healthy granulation and rapid healing’. *C. officinalis* mother tincture is indicated for various septic-gangrenous conditions (Sinha, 1981:78).

Bresler *et al.,* (2007) used the scratch assay to determine the effects of *C. officinalis* 3 cH and PBM on wound healing in combination and singly, however a statistically significant effect was not found, despite encouraging cellular findings. Hostanska *et al*., (2012) investigated the wound healing properties of a homeopathic compound product containing *C. officinalis* 4 X/D (dilution of 10^{-4}), *Arnica montana* 4 X/D, *Hypericum perforatum* 4 X/D and *Symphytum officinale* 6 X/D. They also included groups treated with this product diluted to 1/100 and 1/1000 with hydroethanol, which was either

succussed or not succussed. They used NIH 3T3 fibroblasts and assessed wound healing based on trans-well chamber migration and a commercially available wound healing assay kit using the scratch method. None of the preparations affected cell viability or influenced proliferation; however, fibroblast migration was increased with the non-diluted homeopathic product and it enhanced wound closure.

2.5.4 *Hypericum perforatum* L. *(H. perforatum)*

The major active ingredients found in St. John's wort, or *H. perforatum,* are hypericin and hyperforin, while other constituents include phenylpropanes, flavonol glycosides, biflavones and proanthocyanidins (Nahrstedt & Butterweck, 1997). Research has revealed this plant possesses anti-depressant, anti-viral, anti-bacterial, anti-fungal and anti-cancer activity (Russo *et al*., 2014). It contains various substances that are anti-microbial such as essential oils, phloroglucinols, flavonoids and tannins. It also acts directly on the capsids of viruses by inhibiting their mobility, and it has been shown to decrease the release of pro-inflammatory cytokine IL-6 and to inhibit NF-κB which is implicated in the development of cancer (Russo *et al*., 2014).

H. perforatum is used in phytotherapy for psychiatric disorders such as depression, obsessive compulsive disorder, seasonal affective disorder and also for polyneuropathy, menopause, and herpes infection, and topically for atopic dermatitis, acute and contused injuries, myalgia and first-degree burns (Braun & Cohen, 2010:816-819).

2.5.4.1 Wound healing effects of *H. perforatum*

Several research studies have revealed its efficacy as a wound-healing agent. An olive oil preparation of *H. perforatum* was shown to increase wound healing, a statistically significant effect that was greater than that induced by olive oil alone. Süntar *et al.,* (2010) induced a variety of wounds in rats, namely acetic acid-induced increase in capillary permeability, linear excision and circular excision wounds, and then evaluated wound healing based on tensile strength and histopathological markers of wound healing. The *H. perforatum* preparation increased re-epithelialisation and decreased inflammation.

Han *et al*., (2017) tested the efficacy of *H. perforatum* on healing excisional wounds in rats. They used 50% *H. perforatum* oil cream and assessed wound healing through histological examinations and biochemical measures. Wounds healed faster with *H.*

perforatum use, which was ascribed to its ability to enhance epithelialisation and granulation.

Topical application of *H. perforatum* has also been shown to increase wound healing in human clinical trials. Samadi *et al*., (2010) performed a double-blind, placebo-controlled randomised controlled trial (RCT) to determine the effects of *H. perforatum* on caesarean scars. The participants applied *H. perforatum* ointment to their scars three times a day for 16 days. Wound healing was evaluated based on the REEDA scale (redness, oedema, ecchymosis, discharge, and approximation) which assessed these parameters. *H. perforatum* decreased pruritis and pain, increased wound healing and decreased scar formation.

There is a lack of research on homeopathic preparations of *H. perforatum.* Homeopathic texts indicate its use for 'open, painful wounds' and for 'injuries to parts rich in sentient nerves, particularly fingers and toes' (Hering, 1997:128-129). *H. perforatum* mother tincture is indicated for injury to nervous tissue and the spine, and for punctured and lacerated wounds. It is indicated when the affected parts are excessively painful (Sinha, 1981:87). Mohammadi *et al*., (2012) employed a sciatic nerve transection model in rats to determine the effect of homeopathic *H. perforatum* on peripheral nerve regeneration. *H. perforatum* in 30 cH potency was administered twice daily for a week after the surgical procedure. Rats treated with *H. perforatum* had faster and better regeneration of axons and greater recovery of myelinated fibers compared to sham operation and control groups. A systematic review by Raak *et al*., (2012) concluded that *H. perforatum* is successful for treating dental problems such as post-operative pain, swelling and neuropathic pain, based on expert opinion and single case studies. However, study results are heterogenous, and more rigorous research is needed to draw reliable conclusions.

2.5.5 *Echinacea purpurea* (L.) Moench *(E. purpurea)*

The purple cone flower, or *E. purpurea,* is part of the Asteraceae family and is a perennial medicinal herb (Manayi *et al*., 2015). It is especially well-known for treating cold symptoms, due to its anti-inflammatory and immunostimulatory effects. It has complex chemical constituents including alkamides, ketoalkenes, caffeic acid derivatives, polysaccharides and glycoproteins (Manayi *et al*., 2015).

Its immunomodulatory effects are based on the main mechanisms of phagocyte activation, fibroblast stimulation and enhanced leucocyte mobility secondary to increased respiratory activity (Manayi *et al*., 2015). Research suggests it increases innate immunity and enhances the actions of natural killer cells, polymorphonuclear leucocytes, neutrophils and macrophages. *E. purpurea* extract has potent anti-inflammatory effects, reduces expression of pro-inflammatory cytokines *in vitro* and ameliorates the symptoms of atopic eczema when applied topically (Oláh *et al*., 2017).

The clinical uses of herbal *E. purpurea* are for upper respiratory tract infections, wound healing, herpes infection, reducing chemotherapy side effects, halitosis and recurrent candidiasis. It is commonly used to treat infections and to prevent contracting them (Braun & Cohen, 2010:394-395).

2.5.5.1 Wound healing effects of *E. purpurea*

It has also been investigated in the context of wound healing to some extent, such as in arsenic-induced skin necrosis. Rezaie *et al*., (2013) created wounds in rats using subcutaneous arsenic and tested the effect of *E. purpurea* herbal extract on healing. Histopathological data revealed increased granulation tissue and angiogenesis in the rats that received *E. purpurea* intraperitoneally.

In a clinical trial, Kim *et al*., (2002) tested the effects of a combined herbal extract of *E. purpurea* and *Echinacea angustifolia (E. angustifolia)* on participants over a four-week period. Besides improving their overall physical health and wellbeing, it increased the complement properdin, which could indicate activation of the complement immune system. They also found decreased TNF-α in this group, however, could not draw firm conclusions from these findings due to a large variety in participants' baseline values.

There is a dearth of data on homeopathic preparations of *E. purpurea* in the field of wound research. The use of *E. purpurea* is indicated in homeopathic texts for 'septic conditions in general' and for 'old tibial ulcers' and gangrene, and these indications are the same for the mother tincture (Boericke, 1999:264). One study using an animal model of induced peritonitis found that a D4 (a dilution of 10^{-4}) dilution of *E. angustifolia* had a protective effect against dexamethasone-induced toxicity in peritoneal leucocytes (Pedalino *et al*.,

2004). It also increased counts of neutrophils. There was a small but significant increase in phagocytosis in groups treated with an 'accord of potencies', namely a mixture of different dilutions of *E. angustifolia* (Pedalino *et al*., 2004).

2.6 Photobiomodulation

2.6.1 Description and background

PBM, formerly referred to as low-level laser therapy (LLLT) or phototherapy, is the use of photons at non-thermal irradiance to modify biological activity (Avci *et al*., 2013). Coherent light sources (lasers) or non-coherent light sources such as filtered lamps, LEDs or sometimes both are used for PBM/LLLT. It is referred to as 'low-level' laser therapy to differentiate it from other forms of laser treatment which have a higher power and are used for applications such as ablation, cutting or thermally coagulating tissue (Avci *et al*., 2013). PBM/LLLT does not release energy in the form of heat, sound or vibration. Thus, it causes a non-thermal reaction. In contrast, high-energy lasers cause increases in temperature so severe, they can damage or even vaporise cells (AlGhamdi *et al*., 2012).

Light therapy is by no means a new invention – sunlight has been used for thousands of years by civilisations in Egypt, India and China to treat skin conditions (Barolet, 2008). Niels Ryberg Finsen, a Danish physician and scientist who won the Nobel Prize in Physiology or Medicine in 1903 for his work, rediscovered solar therapy more recently. This event marks the beginning of phototherapy which involves using an artificial radiation source. It was only later, in the late 1960s that Hungarian physician Endre Mester started experimenting with a low-powered ruby laser (694 nm) to investigate the carcinogenic effects of lasers in mice (Barolet, 2008). He was surprised by his results – laser treatment improved hair growth on the animal's shaved back; this was the first instance of PBM which paved the way for a new field of study (Barolet, 2008). Medical research using both lasers and LEDs has grown since that time and is now used for treating various medical conditions.

Red-beam or near-infrared (NIR) lasers, with 600-1 100 nm wavelengths and 1-500 mW output power, are often used for PBM (AlGhamdi *et al*., 2012). The radiation energy consists of a continuous wave or pulsed light with a constant beam of a relatively low energy density (0.04 to 50 J/cm^2). The power of the laser directed at the cells is measured in milliwatts (mW) (AlGhamdi *et al*., 2012). Not all 'low-level' applications of laser are

equal. The success of PBM depends on its wavelength, power, dose and application time (Andrade *et al*., 2014). Doses between 3 and 6 J/cm^2 seem to be more effective, with beneficial effects like stimulating cellular proliferation while doses exceeding 10 J/cm^2 have suppressive or even harmful effects (Andrade *et al*., 2014; AlGhamdi *et al.,* 2012).

2.6.2 Molecular mechanisms of photobiomodulation

The healing effects of PBM are ascribed to its ability to decrease inflammation and pain and promote tissue repair, while preventing tissue damage. Exposing tissues to low levels of red or NIR light can activate or depress a variety of cellular processes such as increasing blood flow and changing the cellular redox state (Avci *et al*., 2013). The mechanisms of action of PBM are not yet fully understood; however, knowledge has been increasing in recent years and several theories exist (Avci *et al*., 2013).

The main hypothesis involves the enzyme COX which forms part of the mitochondrial respiratory chain (De Freitas and Hamblin, 2016). COX is a photo-acceptor (or chromophore) and transduces photosignals in the red and NIR wavelengths of the light spectrum. It is thought that photons from PBM dissociate inhibitory nitric oxide (NO) from COX and in this way increase electron transport, mitochondrial potential and production of ATP (Figure 2.3) (De Freitas and Hamblin, 2016). Many studies have demonstrated that PBM reduces COX expression, which is used as a marker of inflammation (Hamblin, 2017). However, some studies indicate differences in the expression of COX-1 and COX-2; for instance, De Almeida *et al.,* (2011) found that PBM using 1.0 J can protect skeletal muscle against exercise-induced damage and inflammation, and that this effect can be ascribed to increased mRNA expression of COX-1 but decreased expression of COX-2.

Another theory is that light-sensitive ion channels are activated by PBM, which allows calcium entry into the cell. In addition, it has been shown that photon absorption activates various signalling pathways by means of ROS, cyclic adenosine monophosphate (cAMP), NO and calcium which activates transcription factors. This can cause increased expression of genes involved in protein synthesis, cell migration and proliferation, anti-inflammatory pathways, anti-apoptotic proteins and antioxidant enzymes. In addition, various cell types and tissues such as brain, muscle and nerve tissue respond to PBM and it is apparent that stem cells and progenitor cells are especially susceptible to its effects (De Freitas & Hamblin, 2016).

Fibroblasts also exhibit changes in response to PBM. Ayuk *et al.,* (2016) used WS1 fibroblasts to investigate changes in gene expression of cell adhesion molecules after laser irradiation at 830 nm with a dose of 5 J/cm^2. They found that PBM modulated the expression of various genes related to ECM production in both 'diabetic' wounded and 'normal' wounded models, which emphasises the importance of ECM-related molecules in wound healing. These results correlate with this group's previous findings using irradiation at 660 nm (Ayuk *et al.,* 2014) and also in diabetic cells where 25 of 64 genes were significantly down-regulated and 10 were significantly up-regulated following PBM (Houreld *et al.,* 2018). Mignon *et al.,* (2018) found that irradiation with short wavelength-light (≤530 nm) inhibited metabolic activity in dermal fibroblasts, while longer wavelength irradiation (≥590 nm) had a neutral effect. Irradiation with NIR light at 850 nm wavelength and a dosage of 20 J/cm^2 stimulated metabolic activity without an increase in ROS production.

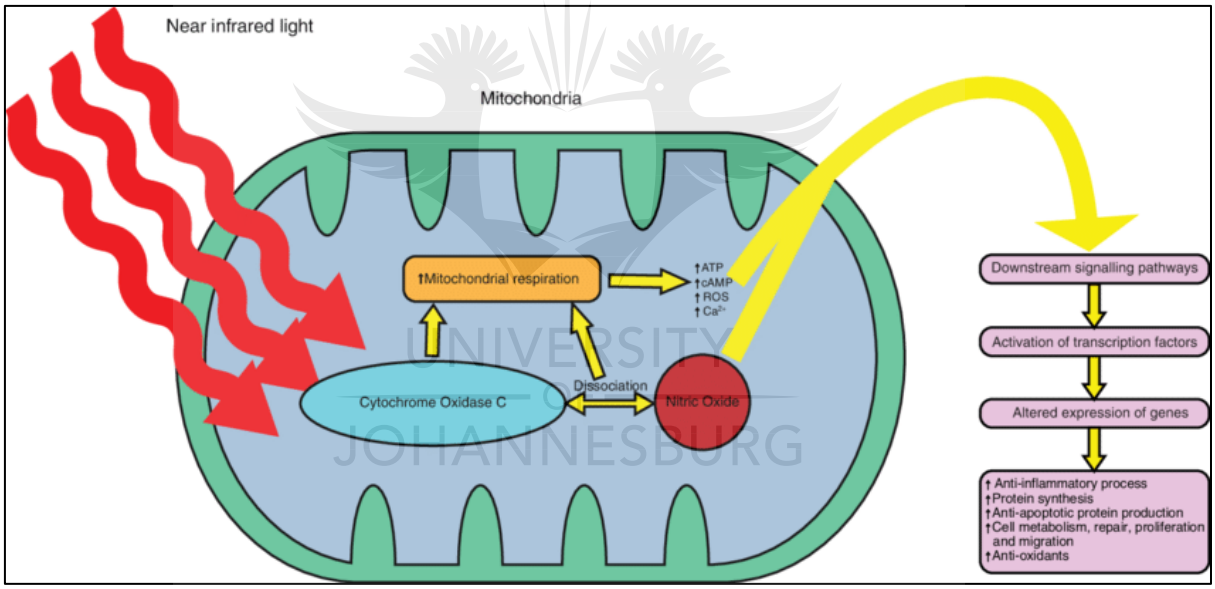

Figure 2.3: The proposed mechanisms of photobiomodulation (Ao *et al.*, 2018).

2.6.3 Photobiomodulation as a therapeutic intervention

PBM is used for medical applications and cosmetically such as for treating fine wrinkles, photo-aged skin, or scars, using a process called photorejuvenation. It is effective in treating a variety of dermatological conditions including acne, burns, hypertrophic scars and herpes virus lesions. The development of LED devices has made PBM more accessible as a treatment option, as they eliminate some of the concerns associated with lasers, namely expense, safety and trained personnel to administer treatment. LED

devices are convenient, and home-use devices are now widely available (Avci *et al*., 2013).

Experimental studies have revealed the wound healing effects of PBM. Al-Watban and Andres (2000) completed *in vitro* studies using a 632.8 nm helium-neon (HeNe) laser on various cell lines and *in vivo* studies using different wavelengths of laser on oval full-thickness wounds in rats. They found that application of laser accelerated wound healing by 29% when animals were treated with a HeNe laser of 632.8 nm. In addition, their cell culture investigations showed laser exposure stimulated, inhibited or had zero effect on the growth of different cell types, dependent on the dose of the laser. However, the levels of laser exposure at which these effects took place differed between cell lines.

Maiya *et al*., (2009) tested the wound healing capacity of a 632.8 nm HeNe laser (3 to 9 J/cm^2) in a diabetic rat model. Circular wounds were made and treated with laser for five days a week until wounds were healed and compared to wounds in rats that received sham irradiation. Wound healing was greatly accelerated by laser treatment, and furthermore this effect was greatest at a dose of 4 to 5 J/cm^2, while doses of 7 to 9 J/cm^2 delayed healing slightly. Laser treatment significantly increased wound contraction and hydroxyproline, a constituent of collagen.

Human trials confirm this data. In an RCT by Hopkins *et al*., (2004), wounds were created on the forearms of 22 participants and subsequently treated with PBM at a wavelength of 820 nm and dosage of 8 J/cm^2. Wounds were monitored at set time intervals until twenty days after injury. They found that PBM facilitated faster wound healing than in the sham group, and participants treated with laser had increased wound contraction (Hopkins *et al*., 2004). A double-blind clinical trial by Ramos *et al*., (2019) showed the healing effects of PBM on post-operative scars. Participants with abdominoplastic scars received ten sessions of 520, 590 and 645 nm wavelength and 10 J/cm^2 dose PBM on the right side of their scars while the left side served as a control. Six months after surgery, scar tissue treated with PBM had significantly improved quality based on the Vancouver Scar Scale, Patient and Observer Scar Assessment Scale and photographic records.

2.6.4 Photobiomodulation for treating diabetic foot ulcers

The healing effect also applies in the context of hyperglycaemia. Houreld *et al*., (2018) created a diabetic wound model by continuously growing fibroblasts in media with added glucose. They found that PBM using irradiation at a wavelength of 660 nm and 5 J/cm^2 dosage modulates gene expression of various cell adhesion molecules, illustrating one mechanism by which laser therapy increases wound healing. In other similar studies, it was found that laser irradiation with 660 nm or 830 nm at a dose of 5 J/cm^2 significantly increased collagen type 1 and TIMP proteins and decreased MMP-3 and MMP-9 in diabetic wounded fibroblasts (Ayuk *et al.,* 2018). Jere *et al.,* (2018) investigated the effects of PBM (660 nm, 5 J/cm^2) in the same model to establish the role of certain signalling pathways in wound healing. They found that PBM significantly increased EGF levels, activation of its receptor (p-EGFR) as well as the Janus kinase/Signal transducer and activators of transcription (JAK/STAT) pathway which together indicates stimulation of cellular proliferation and migration.

Kaviani *et al*., (2011) tested the effect of PBM on chronic, non-ischaemic DFUs and found that it fast-tracked healing. The mean healing time for patients receiving PBM with irradiation of 685 nm wavelength and 10 J/cm^2 dose was 11 weeks compared to 14 weeks in the placebo group, a difference that did not reach statistical significance at this time point. However, at four weeks there was a statistically significant decrease in ulcer size in the PBM group. PBM using a pulsed wave laser at 890 nm irradiation with 0.324 J/cm^2 decreased microbial flora in the wound bed and enhanced the biomechanical properties of wounds in an animal model of Type 2 DM (Asghari *et al.,* 2017). In another animal study of hyperglycaemia, PBM treatment (843 nm wavelength, 5.7 J/cm^2 dosage) significantly decreased the area of inflammatory infiltrates in abdominal adipose tissue in mice over a period of four weeks, as well as significantly decreased blood glucose levels 24 h after irradiation (Yoshimura *et al.,* 2016).

Carvalho *et al*., (2016) investigated the efficacy of *C. officinalis* oil and PBM for treating DFUs in an RCT of 32 patients. They used a 658 nm laser with a dose of 4 J/cm^2 and irradiated the wounds three times weekly for a total of 12 sessions. Wounds were washed with 5 mL *C. officinalis* oil for 30 days in the group receiving *C. officinalis* alone, and in

the combination group, the laser protocol was applied together with *C. officinalis* oil. PBM applied singly or with *C. officinalis* significantly decreased pain and the wound area.

An animal model of diabetes was used to evaluate PBM effects on collagen production and organisation as well as mitochondrial dynamics (Tatmatsu-Rocha *et al*, 2018). PBM up-regulated collagen production, improved angiogenesis as well as VEGF production. It also had beneficial effects on mitochondrial fission-fusion balance and inflammation.

CHAPTER 3

MATERIALS AND METHODS

3.1 Project Approval

This research project was approved by the Higher Degrees Committee of the Faculty of Health Sciences of the University of Johannesburg (clearance number HDC-01-11-2019, Appendix B) and by the Research Ethics Committee of the Faculty of Health Sciences of the University of Johannesburg (Ethics number REC-01-19-2019, Appendix C. The study was conducted at the Laser Research Centre (LRC) (see Appendix D for permission letter) of the University of Johannesburg. Details of materials, reagents and calculations used are shown in Appendix E.

3.2 Experimental Design

A summary of the experimental design can be seen in Figure 3.1. This was a quantitative experimental study using an *in vitro* model.

3.3 Cell Culture

3.3.1 Reconstitution of frozen fibroblasts

Commercially available human skin fibroblasts (WS1, American Type Culture Collection or ATCC® CRL-1502™, Appendix F) were used as an *in vitro* model in this study. Stocks of these cells are available in the LRC and are stored in liquid nitrogen, which allows them to remain viable indefinitely. Frozen stocks, which contained 1 X 10^6 cells, were removed from the liquid nitrogen and thawed by partially submerging the cryovial in a 37°C water bath until a small amount of ice remained. The outside of the vial was sterilised with 70% ethanol (EtOH) and placed inside the Class II biological safety cabinet, in which all tissue culture work was performed to prevent contamination. Cells were added dropwise into a 75 cm^2 flask containing 15 mL pre-warmed supplemented medium using a disposable 2 mL pipette. Cells were incubated for 24 h after which the flask was viewed using an inverted microscope (Wirsam Scientific, Olympus CKX41) to ensure attachment and the medium containing cryoprotectant agent (Biofreeze) removed and replaced with pre-warmed supplemented medium.

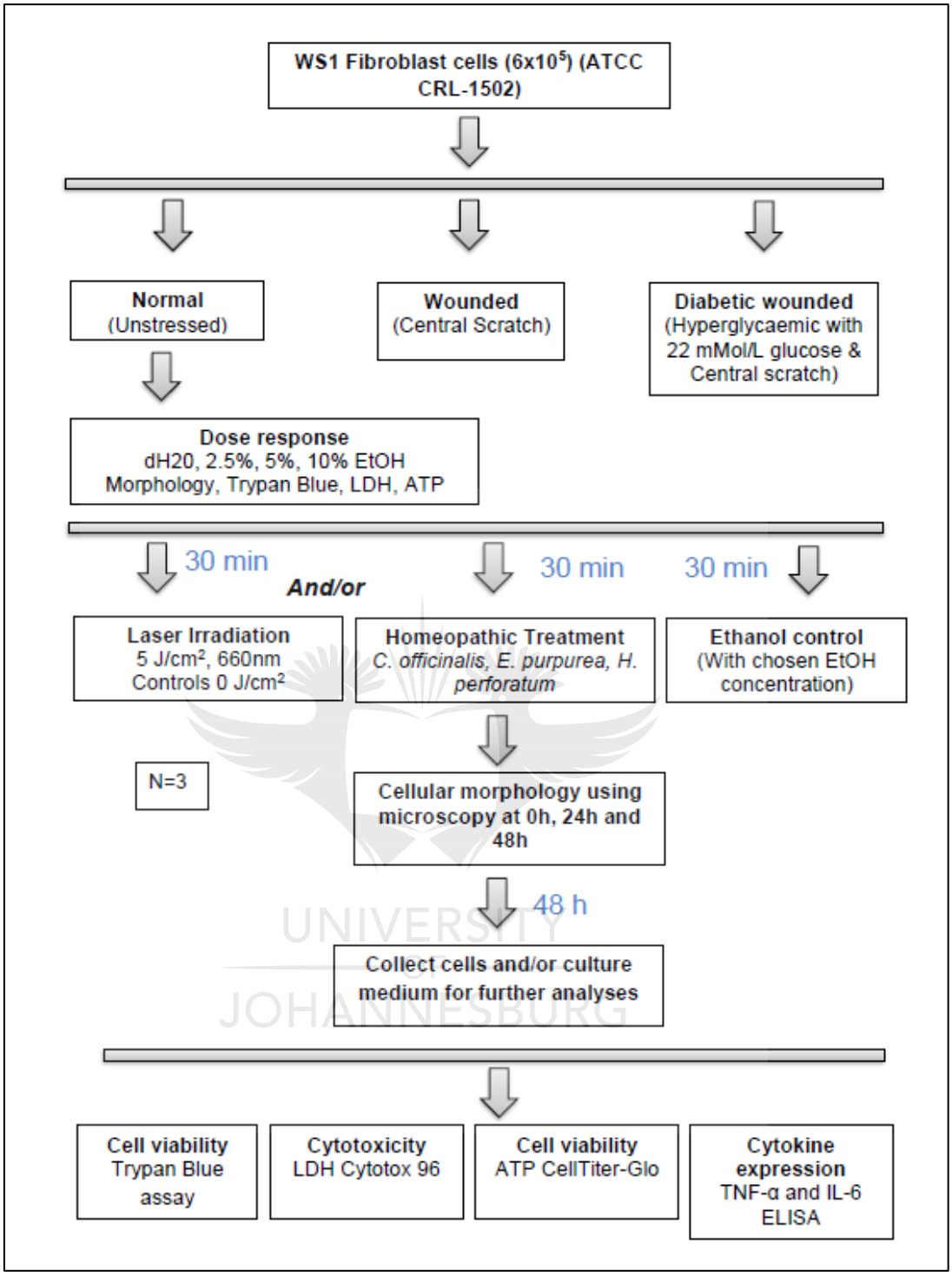

Figure 3.1: Experimental design.

3.3.2 General cell culture

Cells were incubated in an incubator set at 37°C, 5% carbon dioxide (CO_2) and 85% humidity. They were cultured in Minimum Essential Medium (MEM), supplemented with 10% foetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids (NEAA), 1% Penicillin-Streptomycin (100 units Penicillin, 0.1

mg/mL Streptomycin) and 1% Amphotericin-B (2.5 µg/mL) in 75 cm^2 or 175 cm^2 culture flasks. Details of these reagents are shown in Appendix E1. Cells between passage number 11 and 16 were used in experiments.

Flasks were viewed daily using an inverted microscope to determine cell density and to confirm the absence of microbial or other contamination. When a confluence of 70-80% was reached cells were detached and either used for experimental assays, sub-cultured or frozen as stock. Medium was removed from the flask by aspiration and discarded in a waste container with 10% sodium hypochlorite (JIK). The cell monolayer was rinsed with 10 mL pre-warmed Hanks' Balanced Salt Solution (HBSS) and the dissociation agent TrypLE™ Select added (1 mL per 25 cm^2) to detach cells from the flask surface. The flask was incubated at 37°C for 5 min then tapped gently to encourage cells to detach, after which the flask surface was viewed to ensure complete detachment. The cell suspension in TrypLE™ Select was removed and transferred to a 50 mL Falcon tube, and the flask was rinsed with HBSS which was also added and then centrifuged for 5 min at 2 500 rotations per minute (rpm) (Heraeus Megafuge 16R, Thermoscientific). The supernatant was discarded, and cells resuspended in 5 mL pre-warmed HBSS and a cell count performed as described below in the 'Trypan Blue' assay. The cell suspension was centrifuged again for 5 min at 2 500 rpm and further used as described.

3.3.3 Sub-culture of cells

Cells were resuspended in 5 mL pre-warmed supplemented medium after re-centrifugation and transferred to 175 cm^2 flasks containing 50 mL pre-warmed supplemented medium using a 10 mL disposable pipette. The flask was gently swirled to ensure uniform distribution of cells before being sprayed with 70% EtOH and placed in the incubator.

3.3.4 Freezing of cells

Cells were frozen according to the following method to ensure adequate cell stocks. After re-centrifugation the supernatant was discarded, and the cell pellet resuspended in 1 mL of cryoprotective medium (Biofreeze) at a density of 1 X 10^6 cells per mL and transferred to 2 mL cryoprotective vials. Cryovials were transferred to the CoolCell™ freezing container (Biocision) which ensures slow freezing of cells to enhance post-thaw cell

viability, and immediately placed in a -20°C freezer for 1 h, then at -80°C overnight after which vials were transferred to liquid nitrogen for indefinite storage.

3.3.5 Creating 'small plates' for experiments

Cells were seeded into 3.4 cm diameter tissue culture plates for all experimental assays. The supernatant was discarded and cells were resuspended in 5 mL pre-warmed HBSS. The amount of plates that could be seeded from one flask was calculated (Appendix E2.4). Cells were seeded at a density of 6 X 10^5 cells per plate in 3 mL medium and allowed to attach overnight. The next morning the medium was removed, cells rinsed with 500 µL HBSS and 2 mL fresh medium was added to culture plates.

3.4 Cell Models

There were three main cell models: normal (unstressed), wounded (subjected to the scratch assay) and diabetic wounded (grown under hyperglycaemic conditions and subjected to the scratch assay). An extra alcohol control group was added to determine the effects of the vehicle used for the homeopathic solution (see 3.6.4).

3.4.1 Diabetic cell model

A diabetic cell model was achieved by continuously growing cells (37°C, 5% CO_2, 85% humidity)in a hyperglycaemic environment; cells were cultured in complete medium (containing 5.6 mMol/L glucose) with an additional 17 mMol/L D-glucose added to achieve a final glucose concentration of 22.6 mMol/L (Houreld & Abrahamse, 2007). To achieve this, 0.9 mL of the 1 M stock D-glucose solution was added to 49.1 mL of complete medium.

3.4.2 Wounded cell model

Wounded cell models were created by using the 'scratch assay' (Felice *et al*., 2015), where a disposable 1 mL pipette was used to scrape the confluent cell monolayer in a straight line, removing cells in the centre of the plate to create a cell-free 'wound'. Cells were allowed to adjust to the wounding process for 30 min at 37°C, 5% CO_2, 85% humidity before further treatment, if any, was performed.

3.5 Photobiomodulation

Photobiomodulation was completed by using a laser supplied by the CSIR National Laser Centre (NLC) of South Africa. A continuous wave diode laser (FC-655-300-MM2-SMA-1-

0; RGBlase, LLC, California, USA), which emits at a wavelength of 660 nm, was used. The laser parameters are summarised in Table 3.1 below. The temperature of the culture medium in the plates did not drop below 32°C as previously demonstrated (Ayuk, 2012). Non-irradiated cells (0 J/cm^2) were used as controls. Culture plates with a diameter of 3.4 cm were irradiated from above, with the lid of the plate removed and in darkness. Irradiation was achieved via fibre optics, at a distance that created a spot size of exactly the same area as the culture plate.

Table 3.1. Laser parameters.

Light source	Diode laser
Wavelength	660 nm
Emission type	Continuous wave
Power output	100 mW
Power density	11 mW/cm^2
Spot size	9.1 cm^2
Dose	5 J/cm^2
Irradiation time	7 min 34 s
Energy	45.4 J

The exposure time to laser light had to be calculated according to the measured output, which was done before each irradiation (Figure 3.2). A power meter (FieldMate Laser Power Meter, Coherent) was used to measure power output at bench level in the dark, before irradiating cells. The calculation shown below was used to determine exposure time. In the calculation 'X' indicates power output measured in mW.

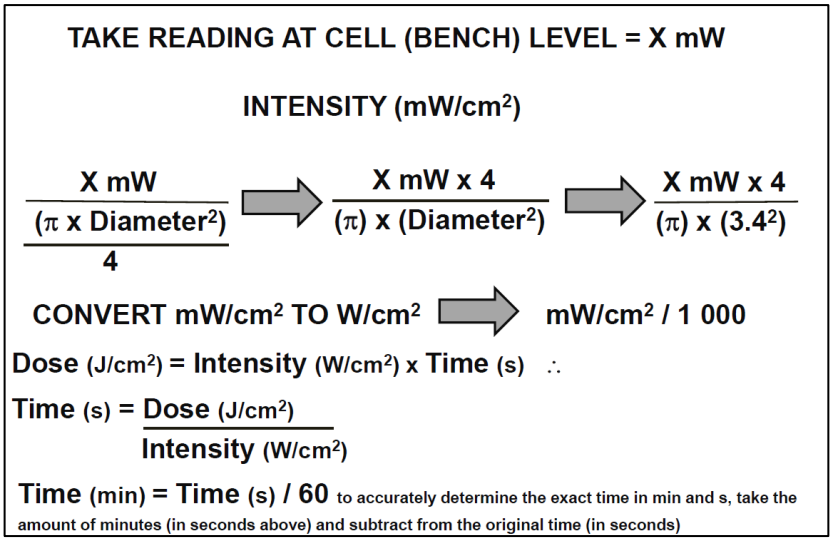

Figure 3.2: Laser exposure time calculation.

Following irradiation, cells were incubated (37°C, 5% CO_2) for 48 h and digital images captured at the 0 h time point (directly following radiation), and at 24 and 48 h thereafter. After 48 h the required amount of culture medium was removed and used for the LDH assay, and the rest frozen at -80°C for performing the ELISA assays at a later stage. Cells were detached, resuspended and used for the Trypan Blue and ATP assays as described below.

3.6 Homeopathic Solution

3.6.1 Determining solvent for homeopathic solution

A preliminary experiment was performed to choose the best solvent to use for the homeopathic solution. As homeopathic remedies are usually prepared in EtOH, a non-toxic effect had to be established before continuing with further experiments. Different concentrations of EtOH were tested as a solvent for the homeopathic solution by using a 3 cH solution prepared in respectively distilled water (0% EtOH), 2.5% EtOH, 5% EtOH and 10% EtOH (see Appendix A for manufacturing methods). These were tested on WS1 fibroblasts according to the methodology of Bresler *et al.,* (2007). For this experiment 6 X 10^5 cells were seeded in 3.4 cm diameter culture plates, the scratch assay was performed, cells allowed to acclimatise for 30 min after which 30 µL of the homeopathic solution in the various concentrations EtOH was added. Cellular morphology, Trypan Blue, LDH membrane integrity and ATP luminescence assays were performed as described below to determine cellular responses.

3.6.2 Homeopathic solution

A 3 cH homeopathic compound solutioncontaining *C. officinalis, H. perforatum* and *E. purpurea* prepared in 5% EtOH was used for the homeopathic solution. The 3 cH solution is a low potency homeopathic medicine which is well-suited to the clinical nature of the treatment of diabetic wounds and has been utilised before in this kind of study (Bresler *et al*., 2007), The solution (30 µL) was added to plates containing 2 mL complete medium using a sterilised pipette tip, which resulted in a final EtOH concentration of 0.074% in a total volume of 2,030 µL . This was done 30 min after the wounding protocol.

3.6.3 Combination treatment

In models receiving both homeopathic and laser treatment, the homeopathic solution was added first as described in 3.6.2, and then cells were irradiated as described in 3.5.

3.6.4 Alcohol control

A 5% EtOH solution was used for the alcohol control models. Cells were wounded and allowed to adjust for 30 min, after which 30 µL of EtOH was added to plates containing 2 mL complete medium, which resulted in a final EtOH concentration of 0.074% in the medium.

3.7 Methods to Determine Cellular Responses

3.7.1 Cellular morphology

Images of the monolayer were obtained at 0, 24 and 48 h after treatment to observe morphological changes in all cell models. The 0 h images were captured directly after irradiation, or after treatment (if any). To achieve this, an inverted light microscope (Wirsam Scientific, CKX41) coupled to a digital camera (Olympus, SC30) was used together with AnalySIS getIT software. Images were taken using the 4X objective, with the plate lid removed and in the dark.

3.7.2 Trypan Blue exclusion assay

This method is based on the principle of dye exclusion – live or viable cells are impermeable to the Trypan Blue dye, whereas dead or non-viable cells take up the dye due to membrane damage. Cells were detached by adding 500 µL pre-warmed TrypLE™ Select to each plate and incubating (37°C, 5% CO_2) for 5 min. Cells were resuspended in 1 mL of serum free medium after centrifugation at 2 500 rpm for 5 min using a bench

centrifuge (Biofuge Pico Heraeus, D37520). Cell suspension (10 µL) was added to a 0.5 mL Eppendorf tube and then 10 µL of 0.4% Trypan Blue was added to stain cells. The mixture was gently mixed by pipetting and then 10 µL added to each chamber of a re-usable plastic cell counter slide, and the sample allowed to settle for 30 s to allow for equal distribution of cells. The Countess® II FL (Invitrogen) was used to obtain the percentage of live cells after ensuring optimal focus and illumination. This instrument captures an image and analyses it to generate the total number of cells, and the number and percentage of viable and non-viable cells. An average of 2 readings was used.

3.7.3 Lactate dehydrogenase (LDH) membrane integrity assay

The CytoTox 96® Non-Radioactive Cytotoxicity Assay was used to quantify LDH. The amount of LDH released reflects the amount of membrane damage and thus cellular toxicity, as normal, non-damaged cell membranes are impermeable to LDH, but damaged membranes allow leakage of LDH into the extracellular fluid (culture media). This is a colorimetric assay where LDH reacts to convert a tetrazolium salt into a red formazan product which can be measured to indicate the number of lysed cells.

Reconstituted substrate mix (50 µL) was added to each well of a clear 96-well plate. Complete medium (50 µL), which had not been used to culture cells in, was added to two wells with substrate mix to serve as a background control. Then 50 µL of culture medium from experimental plates were added to the rest of the wells containing substrate mix. Plate contents were gently mixed by tapping the side of the plate. The plate was covered with foil and incubated in the dark at room temperature for 30 min. Then 50 µL Stop Solution was added to each well, any large air bubbles were burst, and absorbance was detected by using a multiplate reader (Perkin-Elmer, Victor^3, 1420 Multilabel counter) set to 490 nm, with the exposure time set to 1 s. The average background control reading was subtracted from all experimental values.

3.7.4 Adenosine triphosphate (ATP) luminescence assay

The CellTiter-Glo® 3D Cell Viability Assay was used to determine viability in metabolically active cells or viable cells. In this assay thermostable luciferase is used to generate a stable luminescent signal, while inhibiting endogenous enzymes released during cell lysis which can give inaccurate results. The luminescent signal is proportional to the amount

of ATP present, which in turn is directly proportional to the number of viable cells present in culture.

Cells were detached by adding 500 µL pre-warmed TrypLE™ Select to each plate, and incubated (37°C, 5% CO_2) for 5 min. Cells were resuspended in 1 mL of complete culture medium after centrifugation at 2 500 rpm for 5 min using a bench centrifuge (Biofuge Pico Heraeus, D37520). Complete culture medium (50 µL), which had not been used to culture cells in, was added to two wells of an opaque-walled 96-well plate to serve as a background control. Then 50 µL of cell suspension was added to the relevant wells, and 50 µL of the CellTiter-Glo® 3D reagent was added to each well. The plate was covered in foil and placed on an orbital shaker (Polymax 1040, Heidolph Instruments) for 10 min. Luminescence (in Relative Light Units or RLU) was measured using a multiplate reader (Perkin-Elmer, Victor^3, 1420 Multilabel counter). The average background control reading was subtracted from all experimental values.

3.7.5 IL-6 and TNF-α ELISA

The enzyme linked immunosorbent assay (ELISA) is a plate-based methodology used to quantify levels of target molecules such as proteins in samples, by binding to specific antibodies. ELISAs were performed according to the manufacturer's instructions to quantify levels of the pro-inflammatory cytokines IL-6 and TNF-α present in culture media. Both kits are based on the Sandwich-ELISA principle and made use of plates that were pre-coated with antibodies specific to IL-6 and TNF-α respectively. The procedure to complete both assays was the same.

Culture media samples obtained previously were thawed and centrifuged for 20 min at 1 000 g at 8°C. The supernatant was decanted and further used for the assays. Standard working solution (provided) or sample (100 µL of either) was added to each well. The plate was covered and incubated for 90 min at 37°C. The liquid was removed and 100 µL Biotinylated Detection Ab working solution added to each well, the plate was covered, well contents gently mixed and the plate incubated for 1 h at 37°C. The solution was then decanted from wells and 350 µL wash buffer added to each well and discarded, using an automated plate washer (Bio-Rad, PW40). This washing step was repeated twice more. HRP Conjugate working solution (100 µL) was added to wells, the plate covered and incubated for 30 min at 37°C. The solution was decanted, and wells

washed three times as described above. Then 90 µL Substrate Reagent was added to wells, the plate covered and incubated for 15 min at 37°C in the dark. Stop Solution (50 µL) was added to each well and Optical Density (OD) read at once using a plate reader set to 450 nm (Perkin-Elmer, Victor^3, 1420 Multilabel counter). Experimental results were obtained by comparing values to the generated standard curve, after subtracting the standard zero OD value.

The standard curve (Figure 3.3 and 3.4) for IL-6 and TNF-α was generated by calculating the mean OD of the two wells for each of the standard concentrations and then subtracting the mean zero OD value (only sample buffer added to wells) from these values. An XY scatter plot was drawn with concentration on the X-axis and OD on the Y-axis. A line of best fit was inserted, and the equation of the line displayed. The equation, together with the known OD values were used to calculate concentration of protein (IL-6 or TNF-α).

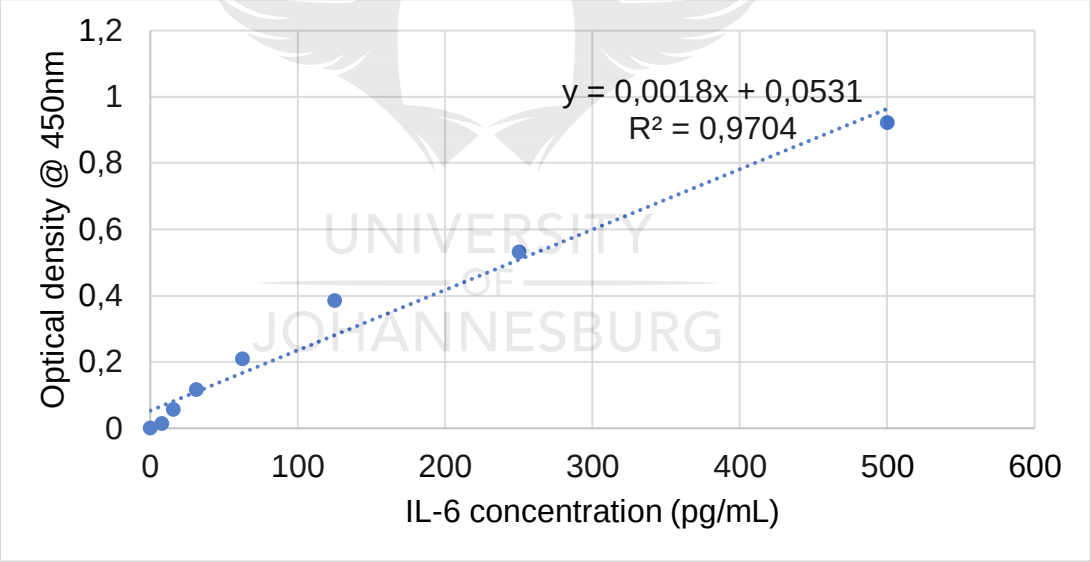

Figure 3.3: IL-6 standard curve (8 point serial standard curve ranging from 0 to 500 pg/mL).

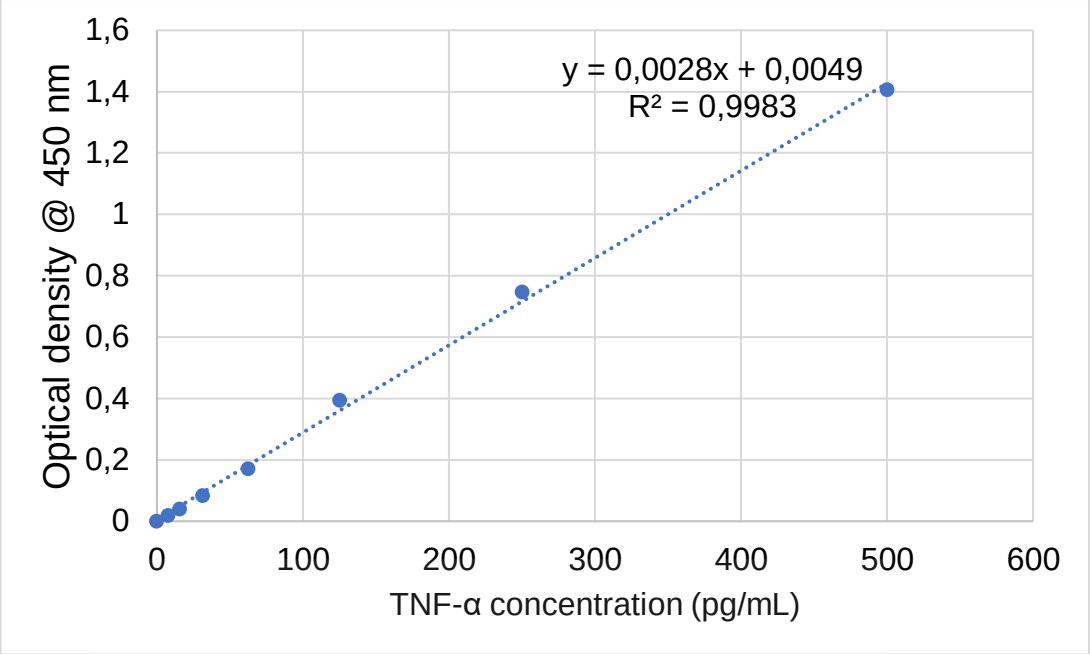

Figure 3.4: TNF-α standard curve (8 point serial standard curve ranging from 0 to 500 pg/mL).

3.8 Reliability and Validity Measures

Measures to ensure quality results were taken; these were to repeat all experiments a minimum of 3 times (n=3) and to include control groups for all the experiments. Assay results were based on duplicate measures where possible. Established experimental protocols were followed according to the research group's and manufacturers' guidelines, and the homeopathic solution as well as the alcohol was manufactured and/or supplied by a reputable homeopathic company (Fusion Homeopathics, South Africa). They manufactured the solution according to established German Homeopathic Pharmacopoeia methods (German Homoeopathic Pharmacopoeia, 2012) and kept detailed records of all steps of the manufacturing process. A commercially available cell line obtained from ATCC were used for all experiments.

3.9 Data Collection and Analysis

Data were captured on the day of the experimental procedure using Microsoft Excel and further data analysis was done using Sigma plot software (version 25). All statistical analyses were completed with the help of a statistician from STATKON (Kuhudzai, 2019). The student t-test was used to determine differences within groups and one-way analysis

of variance (ANOVA) with the Tukey post-hoc test was used to detect differences between group means. *P* values < 0.05 were regarded as statistically significant.

CHAPTER 4

RESULTS

4.1 Determining Solvent for Homeopathic Solution

The best solvent concentration to use for the homeopathic solution had to be determined as a high EtOH concentration may be cytotoxic to the cells *in vitro*. Three different concentrations were tested (2.5%, 5% and 10%) in a normal wounded cell model, and distilled water was used as a control (dH2O). The EtOH was added to culture medium to give final EtOH concentrations of 0.037%, 0.074% and 0.148%, respectively. Cellular morphology, viability (Trypan Blue staining and ATP luminescence) and cytotoxicity (LDH assay) were used to determine the optimal EtOH concentration to employ in further assays.

4.1.1 Cellular morphology

Morphological changes were examined at 0, 24 and 48 h in normal wounded cells incubated with *C. officinalis*, *H. perforatum* and *E. purpurea* prepared in 2.5%, 5% and 10% EtOH using inverted light microscopy. Cells appeared large, flat, and spindle-shaped (stretched), with numerous projections extending out from the ends of the cell body. The central scratch, or 'wound', was visible as an area in the centre of the tissue culture dish with cells on either side. As time progressed, cells could be seen migrating and moving towards the central scratch in all the various models (Figure 4.1). Migration was more rapid over the 48 h incubation period in cells treated with 5% EtOH, with more cells present in the central scratch as compared to those treated with 2.5% and 10% EtOH, and even the dH2O group.

4.1.2 Cellular viability

Cellular viability was determined by Trypan Blue staining and ATP luminescence 24 h after adding the homeopathic solution. There was no significant difference in percentage cell viability (Figure 4.2) and ATP luminescence (Figure 4.3) between the control model (dH2O) and remedies prepared in 2.5%, 5% and 10% EtOH respectively.

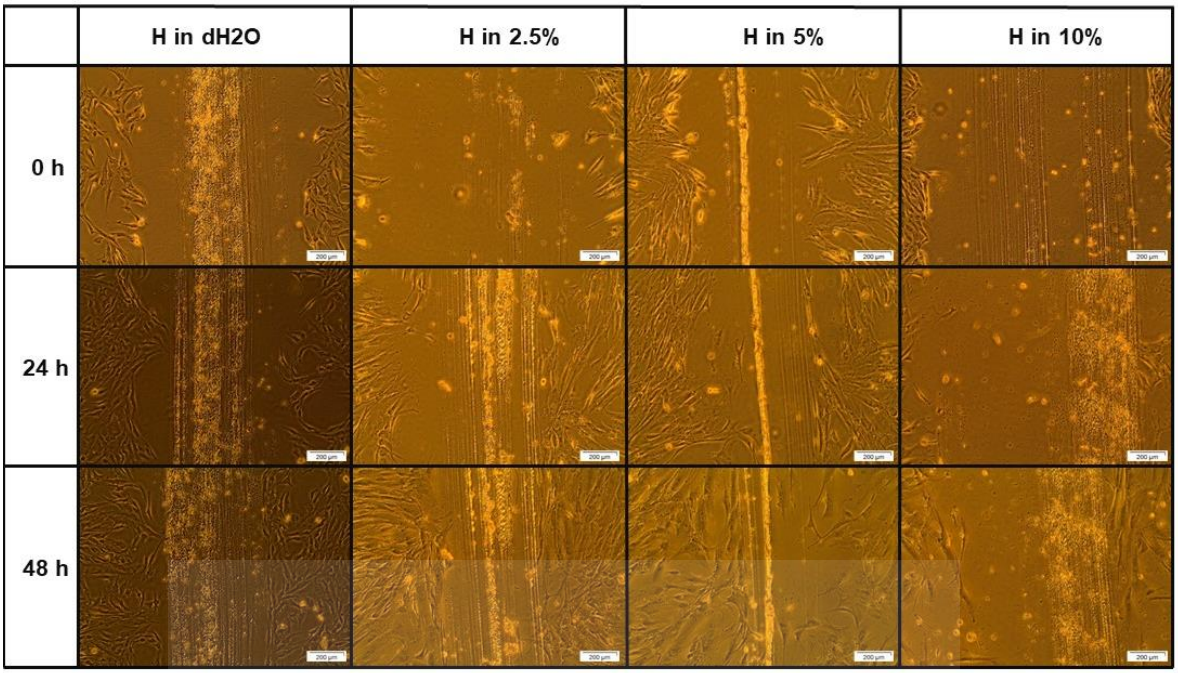

Figure 4.1: Morphology of normal wounded cells treated with homeopathic remedy (H) prepared in different ethanol (EtOH) concentrations (2.5%, 5% and 10%). Cells can be seen migrating into the central scratch (wound), with cells migrating at a more rapid rate when treated with homeopathic solution in 5% EtOH. Scale bars = 200 µm. n=3

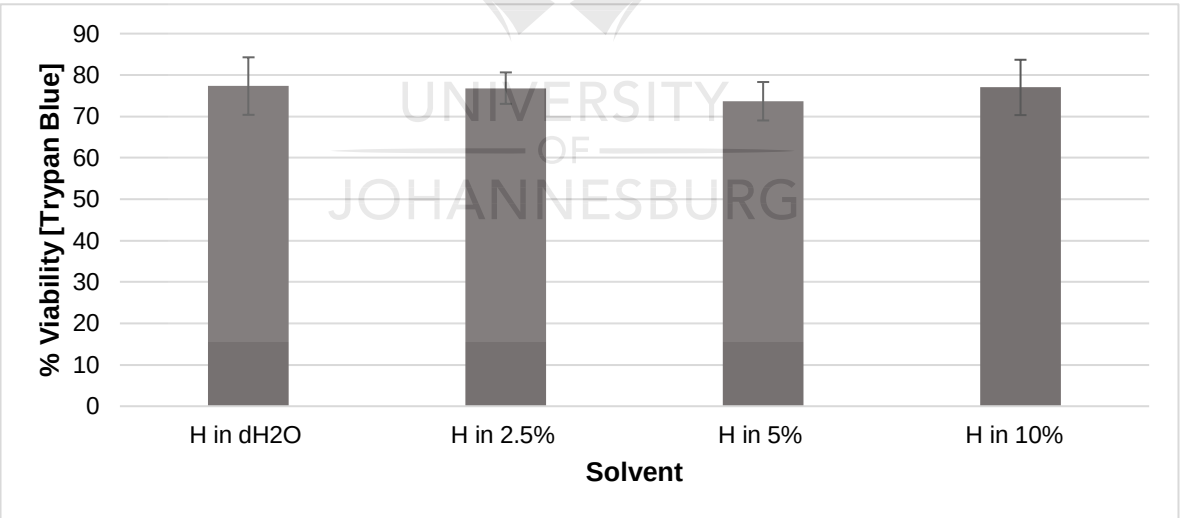

Figure 4.2: Percentage cellular viability as assessed by the Trypan Blue exclusion assay (Mean±SD). Models assessed were normal wounded cells to which homeopathic remedy (H) prepared in distilled water (dH2O), 2.5% ethanol (2.5%), 5% ethanol (5%) and 10% ethanol (10%) was added. No significant differences were found. n=3

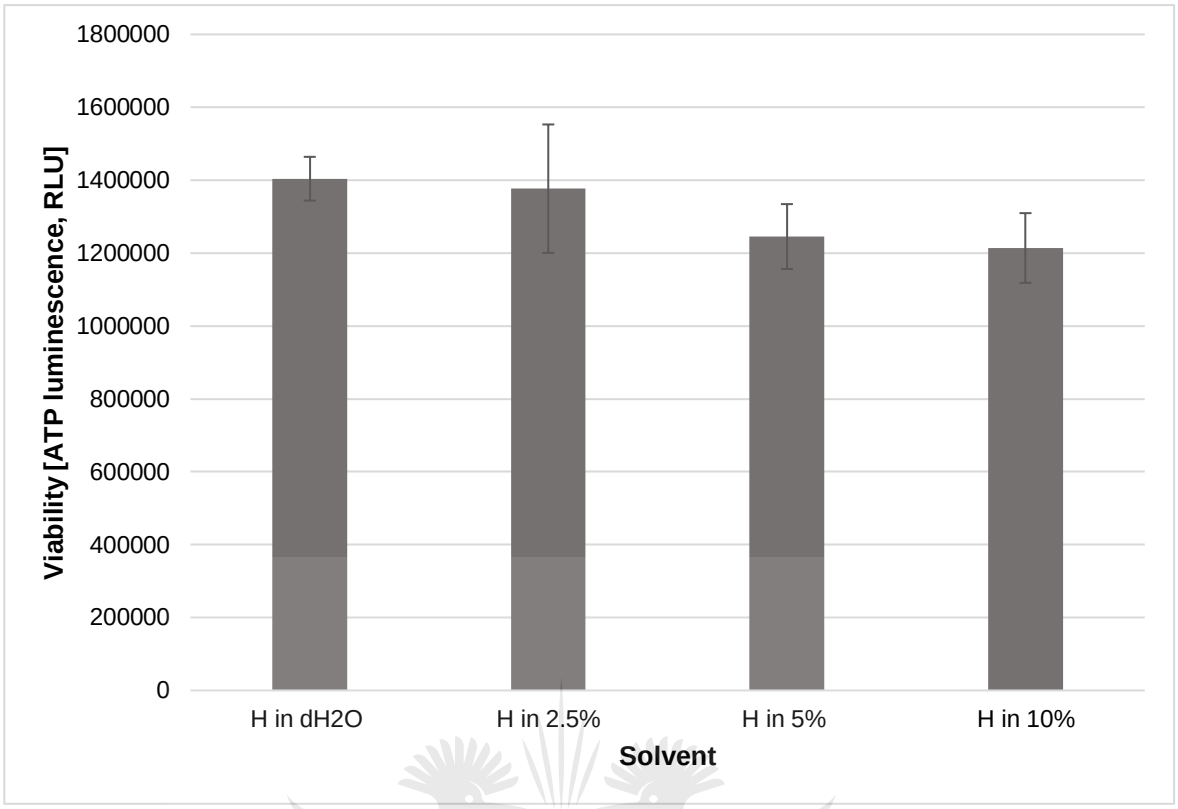

Figure 4.3: Cellular viability was assessed by the ATP luminescence assay (Mean±SD). Models assessed were normal wounded cells to which homeopathic remedy (H) prepared in distilled water (dH2O), 2.5% ethanol (2.5%), 5% ethanol (5%) and 10% ethanol (10%) were added. No significant difference was noted. n=3

4.1.3 Cytotoxicity

Cytotoxicity was determined by measuring LDH which leaked out of damaged cells into the culture media (Figure 4.4). Cytotoxicity data shows there was a significant increase in the release of LDH into the culture media in all three EtOH groups (2.5%, 5% and 10%, respectively) compared to the control (dH2O) group.

Based on these results, an EtOH concentration of 5% was selected as the solvent for the homeopathic solution for further studies. This corresponds with the work of Bresler *et al.* (2007) who also found that an alcohol concentration of 5% was ideal as it produced the greatest degree of cell migration with minimal cell death at 48 h.

4.2 Experiments

4.2.1 Cellular morphology

Cellular responses to the various treatment conditions were determined qualitatively by comparing morphology at 0 h, 24 h and 48 h after wounding. Cell models used were

normal, normal wounded, diabetic wounded and alcohol control (normal wounded cells with 5% EtOH added).

The alcohol control group was added to ensure that the vehicle of the solution did not cause undue stress or prevent wound closure.

Treatment with 5% EtOH by itself (no homeopathic remedies added) increased cellular migration into the cell-free area when compared to the dH2O control (Figure 4.5). This surprising effect was also seen in the preliminary experiment to determine the solution's solvent (see section 4.1). Normal cells which were not wounded (Figure 4.6) did not show any differences following irradiation, homeopathic solution or combination treatment. The cell monolayer remained confluent over the 48 h period.

Irradiating cells induced a dramatic increase in cellular migration in normal wounded groups (Figure 4.7). Homeopathic solution also increased wound closure, however to a lesser degree. Combination treatment had a greater effect on wound closure than either of these conditions alone.

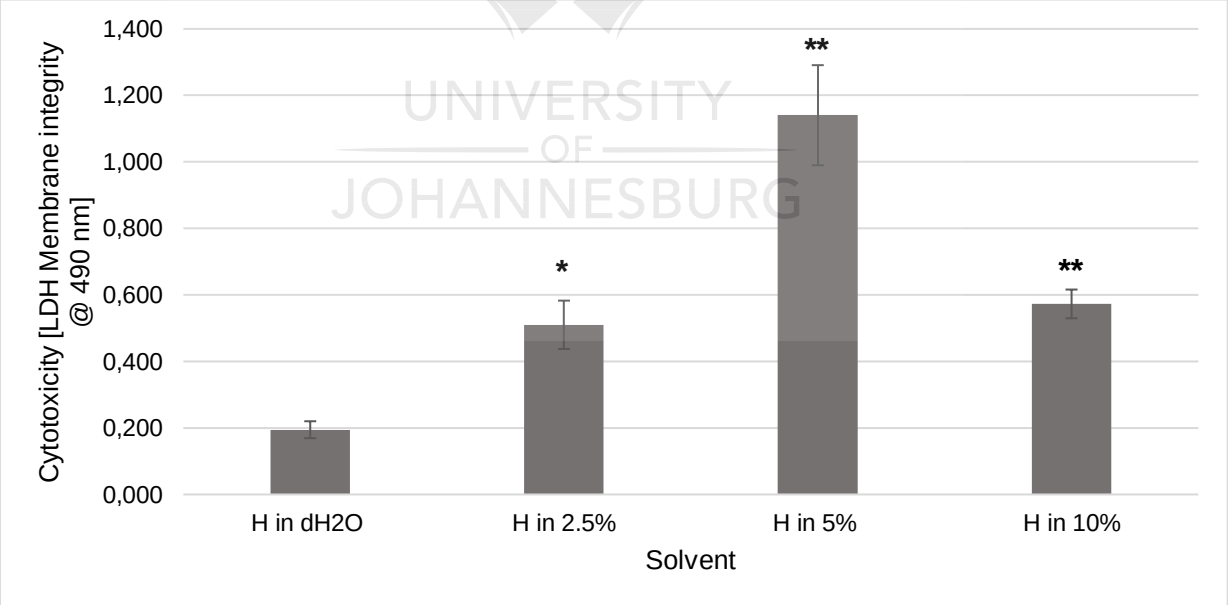

Figure 4.4: Cytotoxicity was assessed by the LDH membrane integrity assay (Mean±SD). Models assessed were normal wounded cells to which homeopathic solution (H) prepared in distilled water (dH2O), 2.5% ethanol (2.5%), 5% ethanol (5%) and 10% ethanol (10%) were added. Significant probability is shown as *P<0.05 and **P<0.01 compared to the control (dH2O) model. n=3

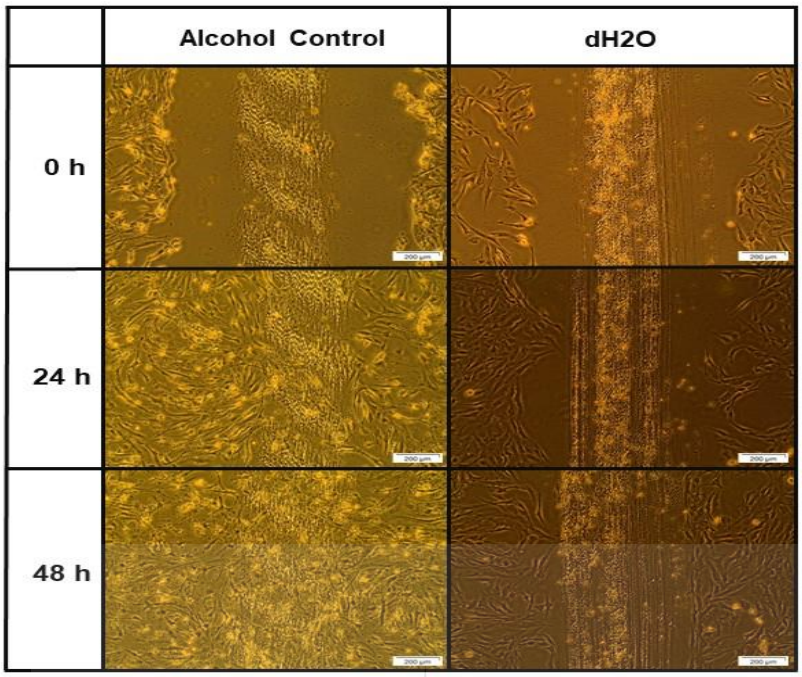

Figure 4.5: Morphology of normal wounded cells treated with 5% ethanol (Alcohol Control) and distilled water (dH2O). Scale bars = 200 µm. n=3

Migration of cells into the central scratch was much less in diabetic wounded models (Figure 4.8) compared to normal wounded models. Cells were present in the wound areas at 48 h but to a lesser extent than in normal wounded cells. Both laser and homeopathic solution improved cell migration in these models compared to the control, and a mild synergistic effect was seen with combination treatment.

Irrespective of the model (diabetic or normal cells) irradiation and homeopathic solution both increased cell migration into the wound area to a greater extent than in controls. Cells maintained their characteristic shape and morphology, and there was minimal detachment from the culture flask and rounding of cells which are indicators of cell stress and cell death.

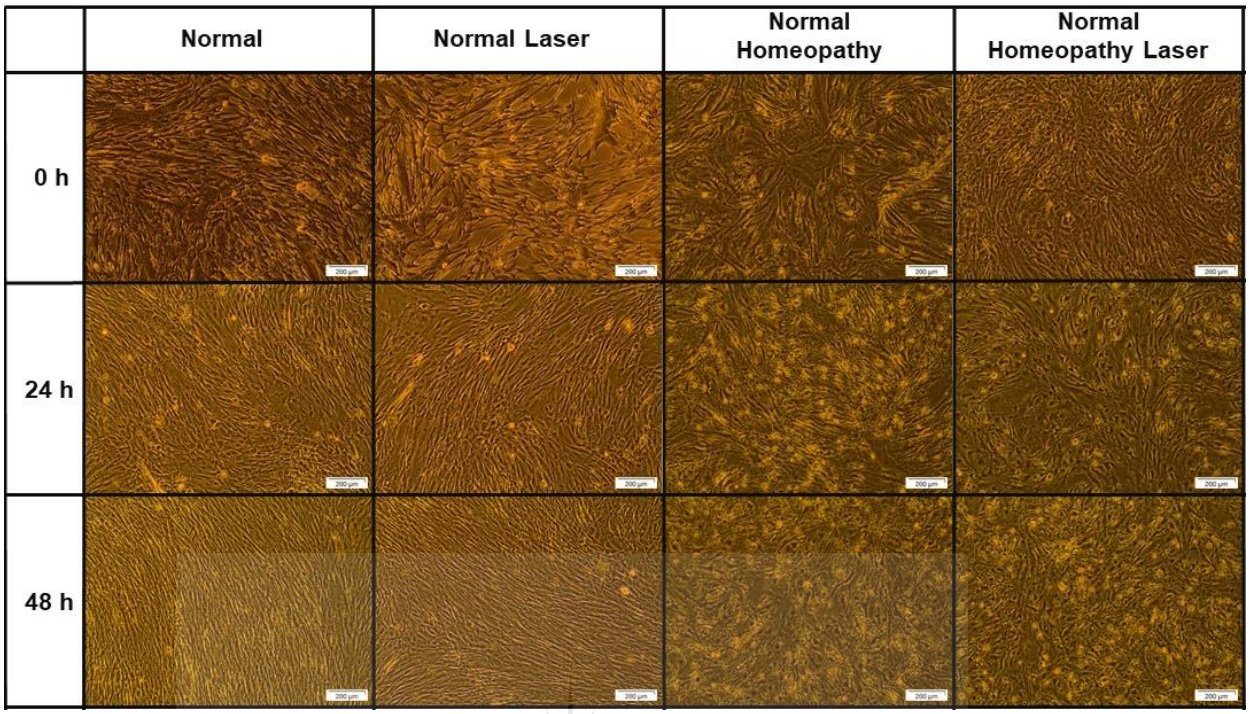

Figure 4.6: Morphology of normal cell models. Treatment with laser irradiation, and or homeopathic solution did not change the morphology of WS1 human skin fibroblast cells. Cultures remained confluent over the 48 h period. Scale bars = 200 µm. n=3

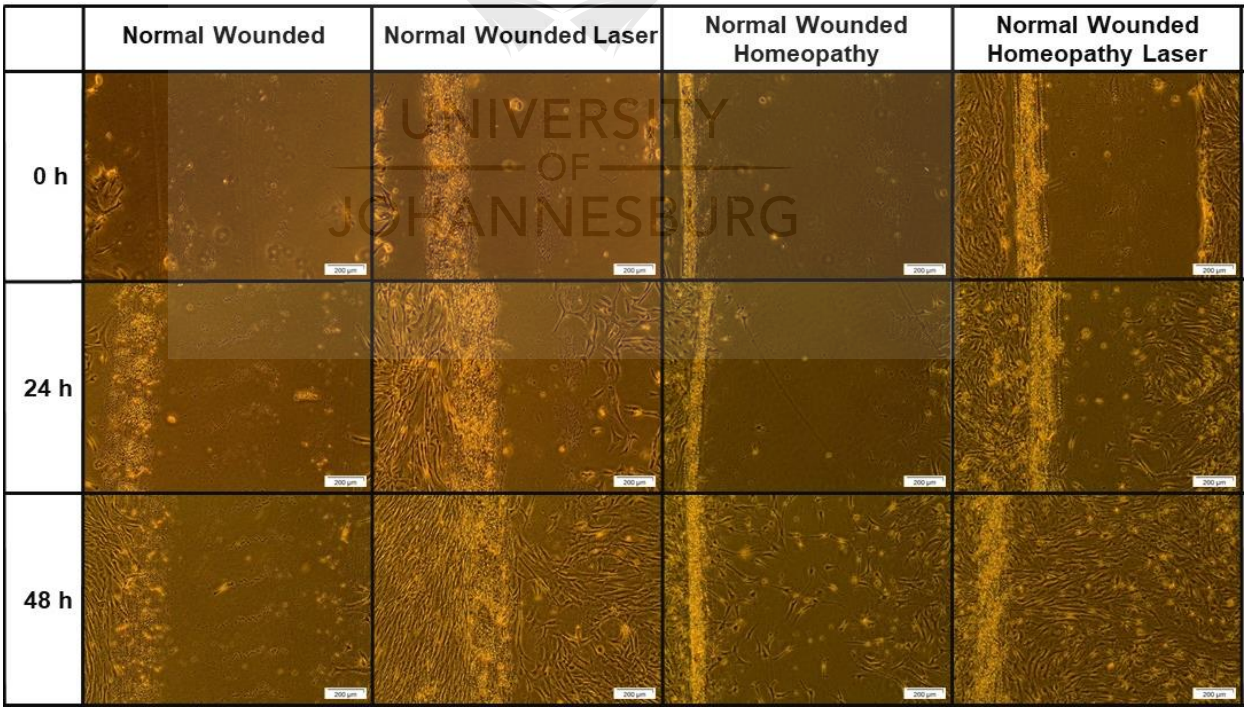

Figure 4.7: Morphology of normal wounded cell models. Laser treatment, homeopathic solution and a combination of the two accelerated cellular migration into the central scratch, with almost complete closure at 48 h in cells treated with both laser irradiation and homeopathic solution. Scale bars = 200 µm. n=3

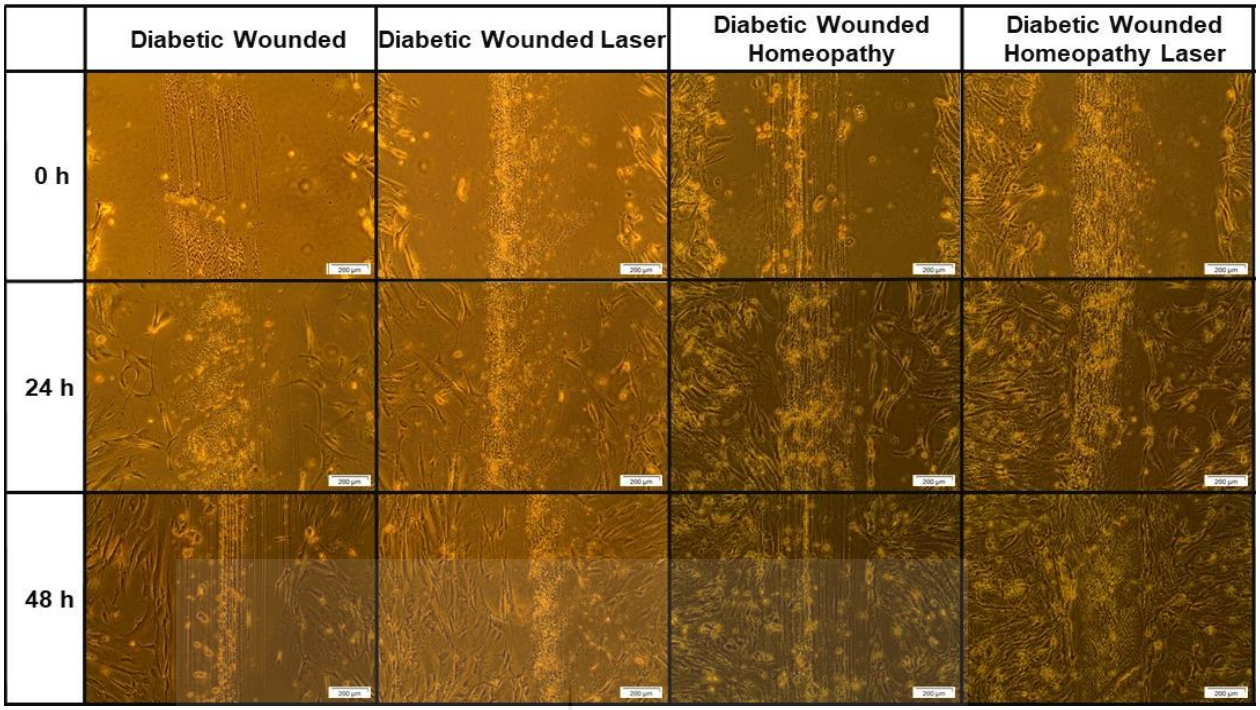

Figure 4.8: Morphology of diabetic wounded cell models. Laser treatment, homeopathic solution and a combination of the two increased cellular migration into the central scratch over a period of 48 h. Scale bars = 200 µm. n=3

4.2.2 Cellular viability

Cellular viability was assessed by the Trypan Blue exclusion assay and by the ATP luminescence assay 48 h post-treatment. In the Trypan Blue exclusion assay (Figure 4.9) all models had a cell viability between 66% (Diabetic Wounded Homeopathy Laser) and 81% (Normal Homeopathy Laser). In normal models the only significant difference was seen in irradiated normal cells which had decreased viability compared to the normal control ($P<0.05$). In the normal wounded models, the cells that received laser irradiation and homeopathic solution had decreased viability compared to the control cells (normal wounded) ($P<0.01$). A combination therapy also resulted in decreased viability in diabetic wounded cells ($P<0.05$). There were no statistically significant differences between group means as determined by one-way ANOVA.

The ATP luminescence results are shown in Figure 4.10. ATP levels were decreased in wounded cells treated with 5% EtOH compared to control cells suggesting that the solvent of the solution had some degree of toxic effect. In normal models, laser treatment and homeopathic solution increased ATP levels in comparison to the control ($P<0.05$). The increase was greater in irradiated cells. However, combination treatment caused a decrease in ATP levels ($P<0.05$). In normal wounded models, laser treatment slightly

decreased viability (P<0.05) while homeopathic solution increased viability (P<0.001). Combination treatment decreased ATP levels (P<0.05). In diabetic wounded models the combination treatment group caused a significant increase in ATP levels compared to the control (P<0.05).

One-way ANOVA revealed statistically significant differences between means in ATP luminescence. Homeopathic solution combined with irradiation levels were lower than irradiation by itself (Normal Laser vs Normal Homeopathy Laser, P<0.001). Wounding significantly decreased ATP levels in normal irradiated models (Normal Laser vs Normal Wounded Laser, P<0.001). In normal wounded models homeopathic solution increased ATP to a significantly greater extent than irradiation (Normal Wounded Laser vs Normal Wounded Homeopathy, P<0.001). Combination treatment also had decreased ATP compared to homeopathic solution (Normal Wounded Homeopathy Laser vs Normal Wounded Homeopathy, P<0.001). The alcohol control group had significantly lower levels of ATP compared to the homeopathic treated group (Normal Wounded (5%) vs Normal Wounded Homeopathy, P<0.001).

4.2.3 Cytotoxicity

Cytotoxicity was assessed by measuring the levels of LDH in culture medium (Figure 4.11). LDH levels were significantly increased by treating cells with 5% EtOH compared to non-irradiated normal wounded controls (P<0.001). In normal models, homeopathic solution increased cytotoxicity (P<0.05) while combining homeopathic solution and irradiation resulted in decreased cytotoxicity (P<0.01). Irradiation of normal cells caused a slight decrease in LDH levels, but this did not reach statistical significance (P=0.072). In normal wounded models a similar trend is observed; homeopathic solution increased LDH levels (P<0.01) and a combination of irradiation and homeopathic solution decreased cytotoxicity (P<0.01). Once again irradiation by itself decreased cytotoxicity but this difference does not reach statistical significance (P=0.093). In diabetic wounded models the situation is reversed; irradiation significantly increased cytotoxicity (P<0.05), homeopathic solution decreased it (P<0.01) but similar to normal wounded models, combination treatment decreased cytotoxicity (P<0.01).

One-way ANOVA showed statistically significant differences between means in LDH levels. In normal models, cells treated with a combination of laser and homeopathic

solution had decreased LDH levels compared to homeopathic solution alone (Normal Homeopathy Laser vs Normal Homeopathy, P<0.001). In normal wounded models, homeopathic solution caused significantly more LDH release than irradiation (Normal Wounded Homeopathy vs Normal Wounded Laser, P<0.001), combination treatment decreased LDH release compared to both irradiation (Normal Wounded Homeopathy Laser vs Normal Wounded Laser, P<0.05) and homeopathic solution (Normal Wounded Homeopathy Laser vs Normal Wounded Homeopathy, P<0.001) by themselves. In diabetic wounded groups combination treatment caused decreased LDH levels as compared to irradiation alone (Diabetic Wounded Homeopathy Laser vs Diabetic Wounded Laser, P<0.001).

4.2.4 IL-6 and TNF-α ELISA

Levels of the pro-inflammatory cytokines IL-6 and TNF-α in culture medium were established by using commercially available ELISA assay kits. The results of the IL-6 ELISA are shown in Figure 4.12. Treating cells with 5% alcohol significantly increased IL-6 concentration compared to non-irradiated normal wounded cells (P<0.05). In normal models, homeopathic solution and combination treatment (homeopathic solution and laser irradiation) increased IL-6 concentration (P<0.05 and P<0.01, respectively). In normal wounded models, irradiation significantly decreased IL-6 concentration (P<0.05), while homeopathic solution by itself increased IL-6 (P<0.05). Combination treatment of normal wounded cells with homeopathic solution and laser irradiation significantly decreased IL-6 concentration (P<0.01). Irradiation of diabetic wounded models significantly decreased IL-6 concentration (P<0.001).

One-way ANOVA revealed statistically significant differences in IL-6 concentration between groups. Wounding produced an increase in IL-6 (Normal Wounded vs Normal, P<0.001). Irradiation decreased IL-6 concentration in the combination treatment group compared to homeopathic solution by itself (Normal Homeopathy Laser vs Normal Homeopathy, P<0.001). In normal wounded models, homeopathic solution caused a significantly greater increase in IL-6 concentration compared to irradiation (Normal Wounded Homeopathy vs Normal Wounded Laser, P<0.001). Combination treatment models had decreased IL-6 concentration compared to the homeopathic group (Normal Wounded Homeopathy Laser vs Normal Wounded Homeopathy, P<0.001). In diabetic wounded models, homeopathic solution once again caused increased IL-6 concentration

compared to irradiation (Diabetic Wounded Homeopathy vs Diabetic Wounded Laser, P<0.001). Combination treatment caused greater IL-6 release than either condition by itself (Diabetic Wounded Homeopathy Laser vs Diabetic Wounded Laser, P<0.001; Diabetic Wounded Homeopathy Laser vs Diabetic Wounded Homeopathy, P<0.05).

Results of the αα ELISA are shown in Figure 4.13. ELISA-TNF-α was decreased by 5% ethanol application (P<0.05). Homeopathic solution in normal cells and combined with irradiation decreased TNF-α concentration (P<0.05). In normal wounded models, homeopathic solution decreased TNF-α concentration as well (P<0.01). In diabetic wounded models, homeopathic solution combined with irradiation significantly increased TNF-α release into culture media (P<0.01).

One-way ANOVA revealed statistically significant differences in TNF-α concentration between groups. Wounding cells decreased TNF-α release (Normal vs Normal Wounded, P<0.05). Combination treatment decreased TNF-α concentration compared to laser treatment alone (Normal Homeopathy Laser vs Normal Laser, P<0.05). Wounding cells also decreased TNF-α concentration in irradiated normal wounded models (Normal Wounded Laser vs Normal Laser, P<0.01). In normal wounded models irradiating cells caused greater TNF-α release compared to homeopathic solution alone (Normal Wounded Laser vs Normal Wounded Homeopathy, P<0.01).

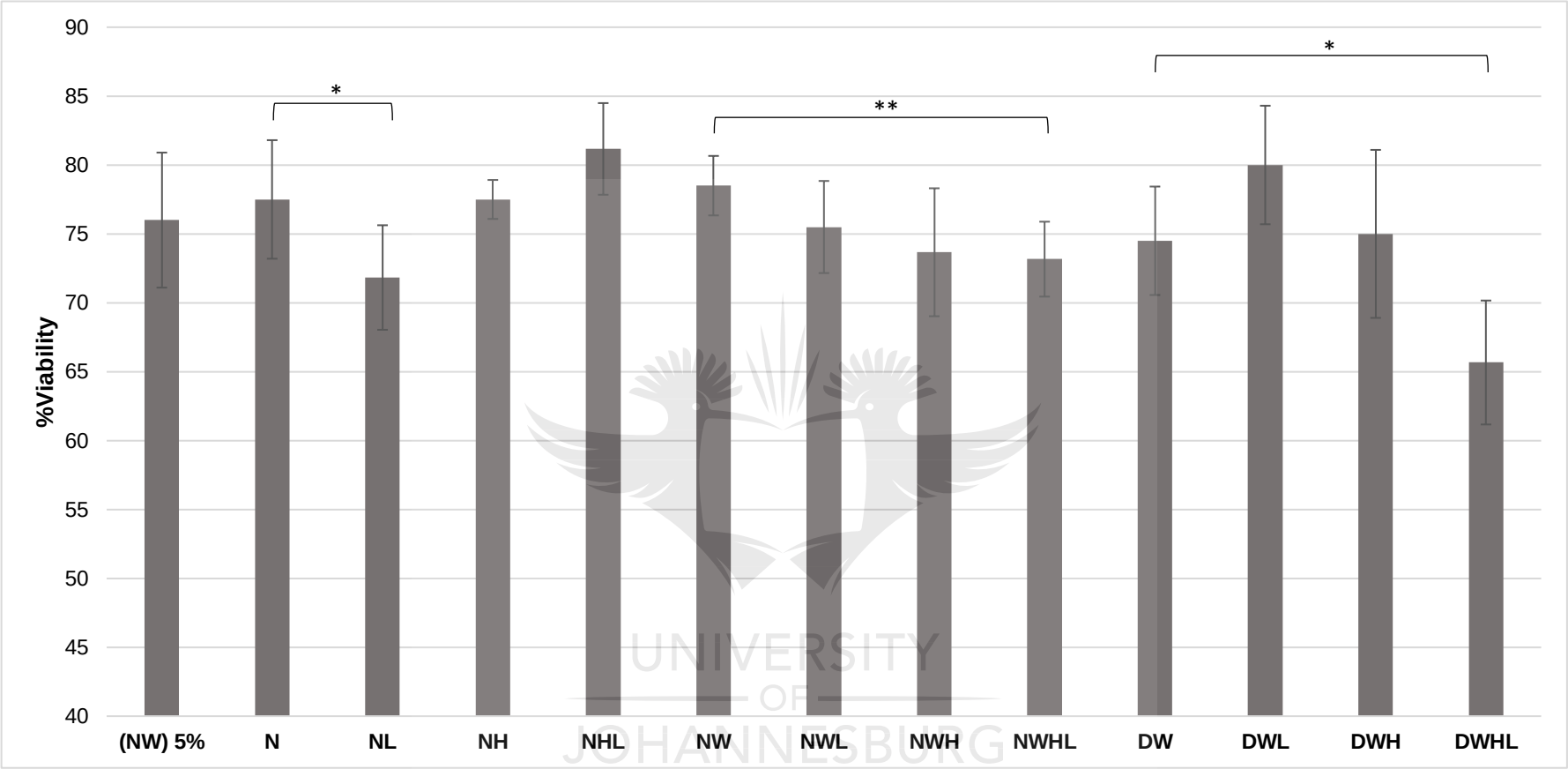

Figure 4.9: Percentage cellular viability was assessed by the Trypan Blue exclusion assay. Models assessed included: normal (N), normal irradiated (NL), normal cells treated with homeopathic solution (NH), normal cells treated with homeopathic solution and irradiated (NHL), normal wounded (NW), normal wounded irradiated (NWL), normal wounded cells treated with homeopathic solution (NWH), normal wounded cells treated with homeopathic solution and irradiated (NWHL), diabetic wounded (DW), diabetic wounded irradiated (DWL), diabetic wounded cells treated with homeopathic solution (DWH), and diabetic wounded cells treated with homeopathic solution and irradiated (DWHL). An alcohol control model was included which consisted of normal wounded cells to which 5% EtOH was added ((NW)5%). Significant probability is shown as *P<0.05 and **P<0.01 as compared to the non-irradiated controls (Mean±SD). n=3

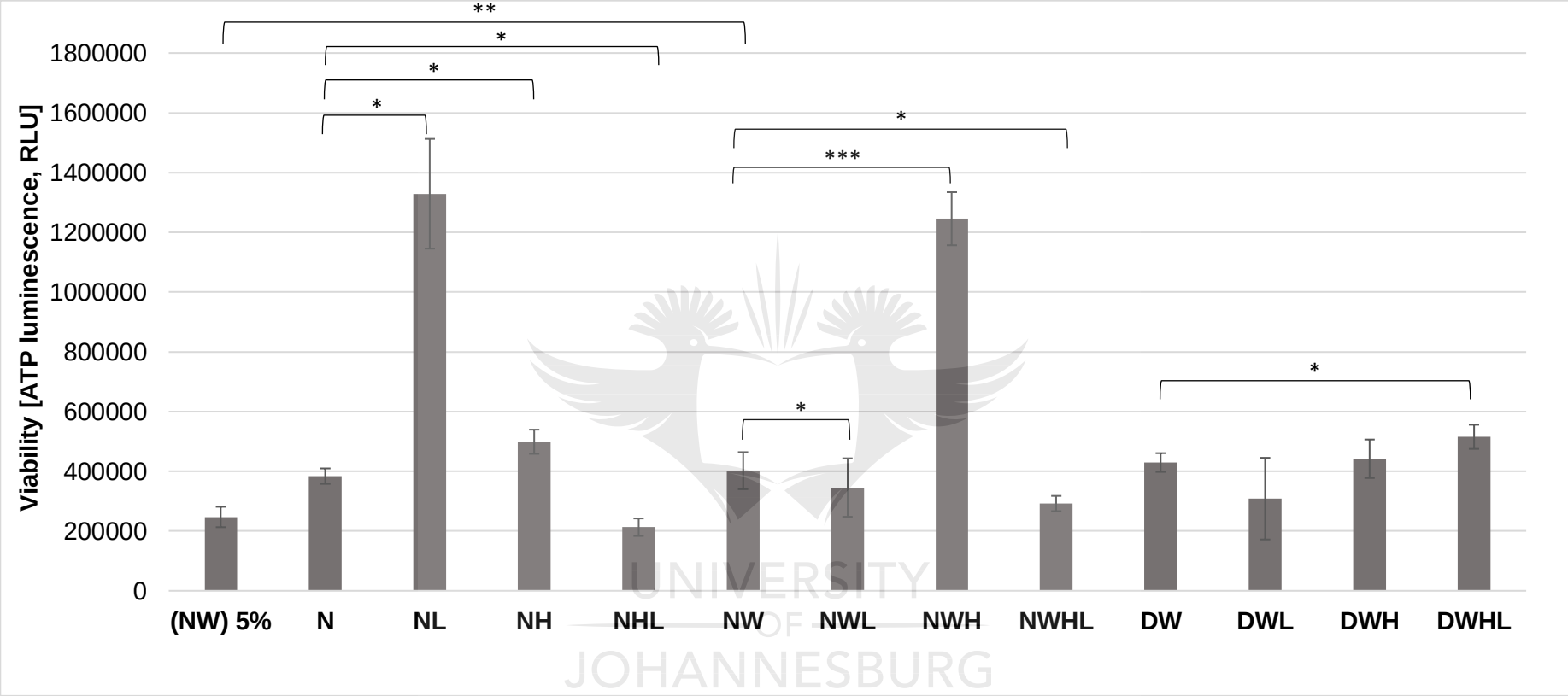

Figure 4.10: Cellular viability was assessed by ATP luminescence. Models assessed included: normal (N), normal irradiated (NL), normal cells treated with homeopathic solution (NH), normal cells treated with homeopathic solution and irradiated (NHL), normal wounded (NW), normal wounded irradiated (NWL), normal wounded cells treated with homeopathic solution (NWH), normal wounded cells treated with homeopathic solution and irradiated (NWHL), diabetic wounded (DW), diabetic wounded irradiated (DWL), diabetic wounded cells treated with homeopathic solution (DWH), and diabetic wounded cells treated with homeopathic solution and irradiated (DWHL). An alcohol control model was included which consisted of normal wounded cells to which 5% EtOH was added ((NW)5%). Significant probability is shown as *P<0.05, **P<0.01 and ***P<0.001 as compared to the non-irradiated controls (Mean±SD). n=3

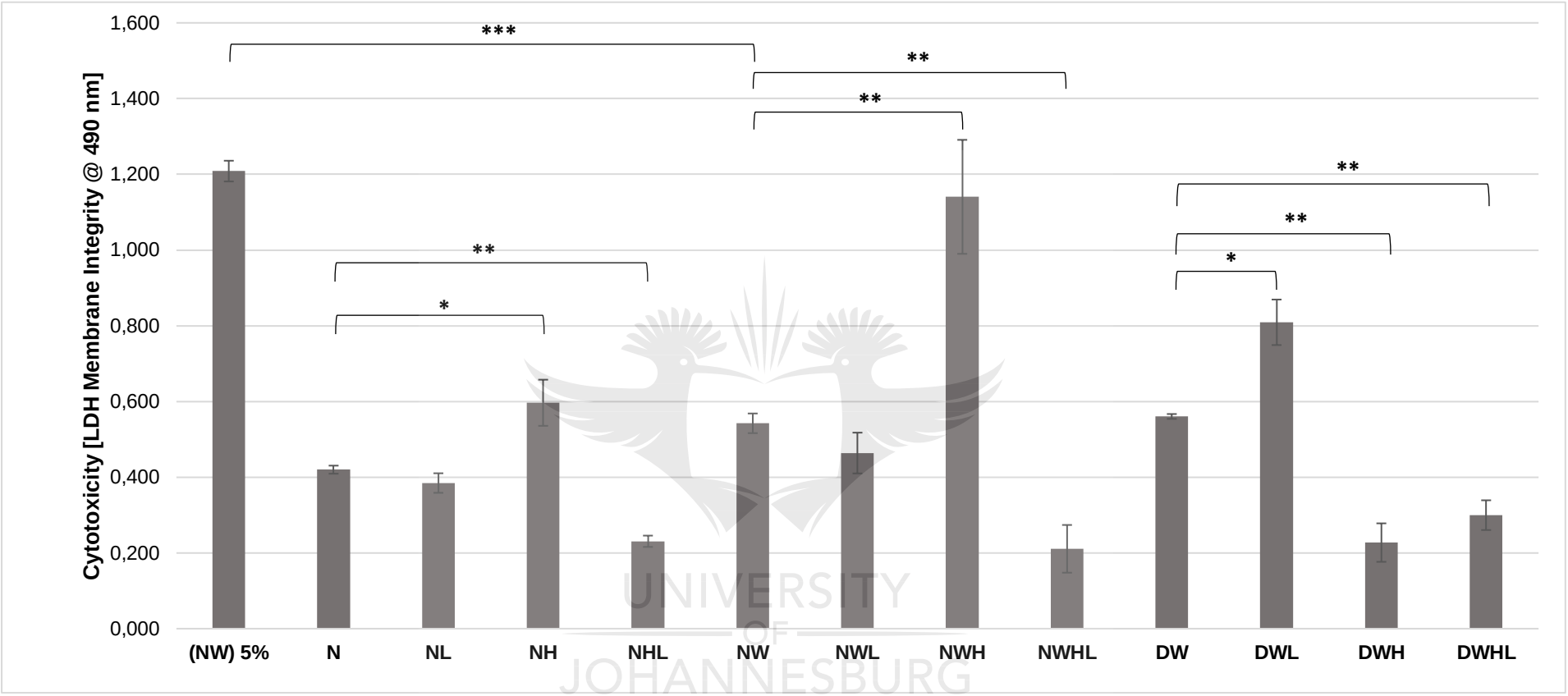

Figure 4.11: Cytotoxicity was assessed by LDH membrane integrity. Models assessed included: normal (N), normal irradiated (NL), normal cells treated with homeopathic solution (NH), normal cells treated with homeopathic solution and irradiated (NHL), normal wounded (NW), normal wounded irradiated (NWL), normal wounded cells treated with homeopathic solution (NWH), normal wounded cells treated with homeopathic solution and irradiated (NWHL), diabetic wounded (DW), diabetic wounded irradiated (DWL), diabetic wounded cells treated with homeopathic solution (DWH), and diabetic wounded cells treated with homeopathic solution and irradiated (DWHL). An alcohol control model was included which consisted of normal wounded cells to which 5% EtOH was added ((NW)5%). Significant probability is shown as *P<0.05, **P<0.01 and ***P<0.001 as compared to the non-irradiated controls (Mean±SD). n=3

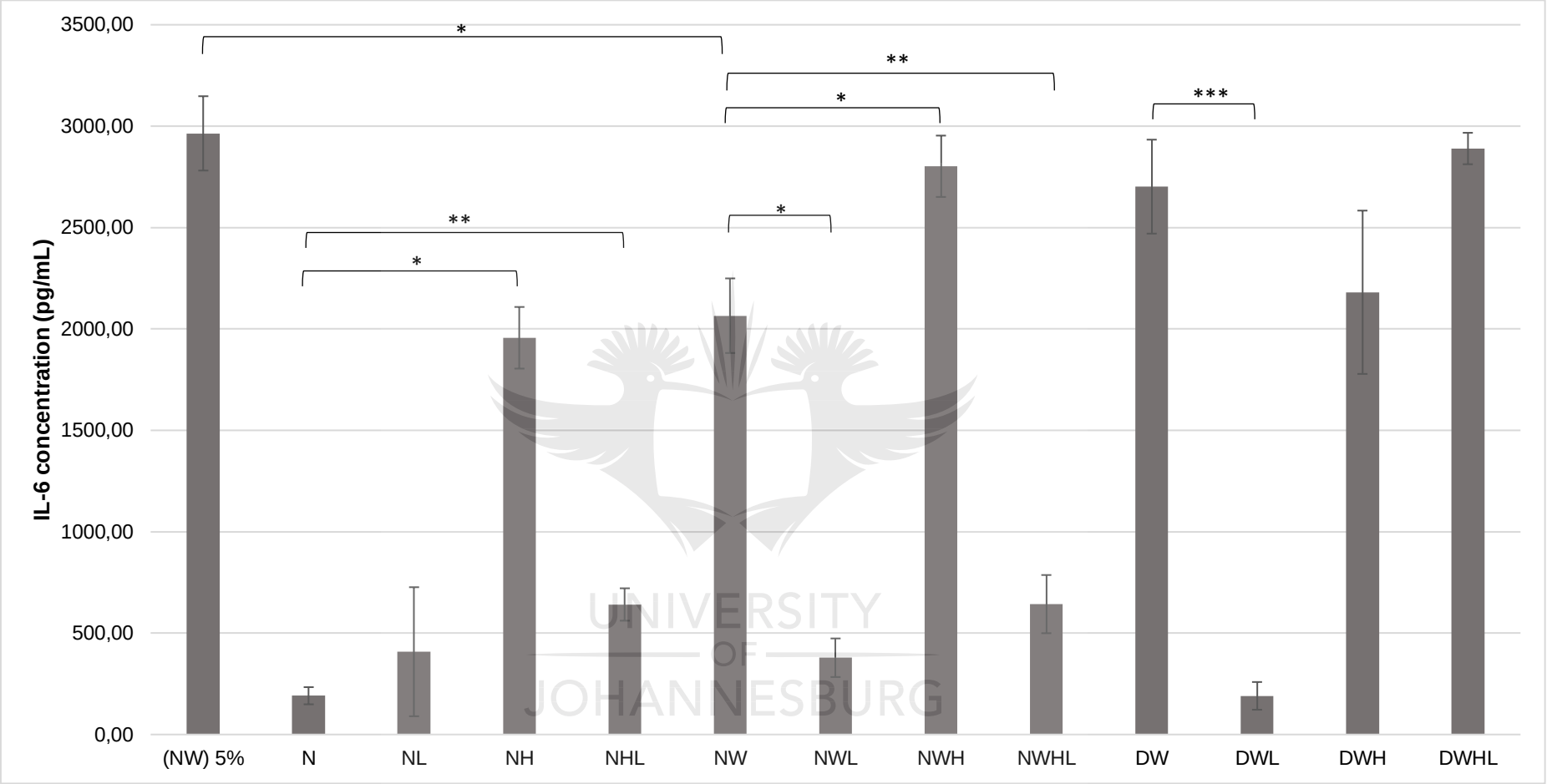

Figure 4.12: IL-6 cytokine levels were tested in culture medium. Models assessed included: normal (N), normal irradiated (NL), normal cells treated with homeopathic solution (NH), normal cells treated with homeopathic solution and irradiated (NHL), normal wounded (NW), normal wounded irradiated (NWL), normal wounded cells treated with homeopathic solution (NWH), normal wounded cells treated with homeopathic solution and irradiated (NWHL), diabetic wounded (DW), diabetic wounded irradiated (DWL), diabetic wounded cells treated with homeopathic solution (DWH), and diabetic wounded cells treated with homeopathic solution and irradiated (DWHL). An alcohol control model was included which consisted of normal wounded cells to which 5% EtOH was added ((NW)5%). Significant probability is shown as *P<0.05, **P<0.01 and ***P<0.001 as compared to non-irradiated control cells (Mean±SD). n=3

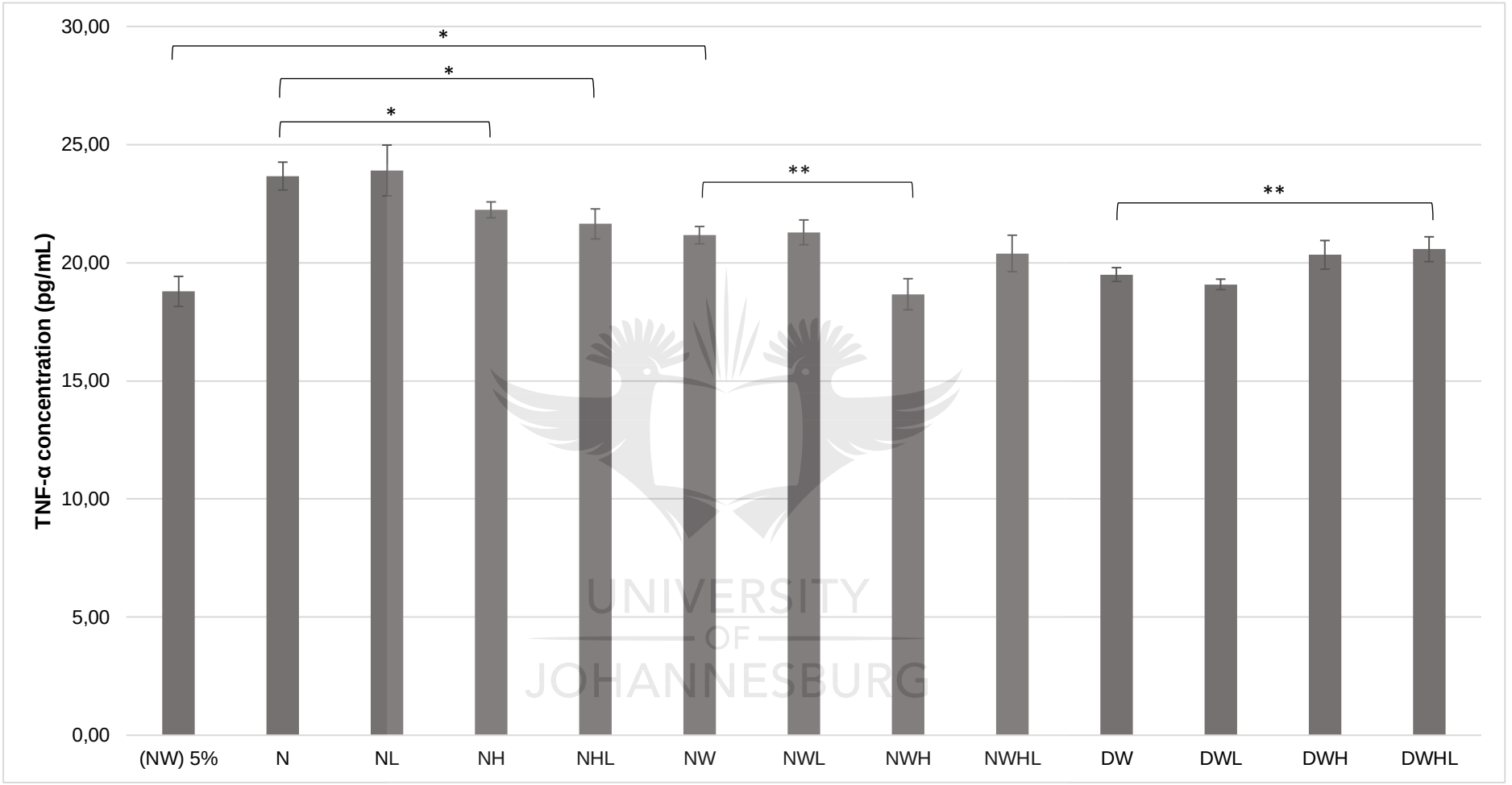

Figure 4.13: TNF-α cytokine levels were tested in culture medium. Models assessed included: normal (N), normal irradiated (NL), normal cells treated with homeopathic solution (NH), normal cells treated with homeopathic solution and irradiated (NHL), normal wounded (NW), normal wounded irradiated (NWL), normal wounded cells treated with homeopathic solution (NWH), normal wounded cells treated with homeopathic solution and irradiated (NWHL), diabetic wounded (DW), diabetic wounded irradiated (DWL), diabetic wounded cells treated with homeopathic solution (DWH), and diabetic wounded cells treated with homeopathic solution and irradiated (DWHL). An alcohol control model was included which consisted of normal wounded cells to which 5% EtOH was added ((NW)5%). Significant probability is shown as *P<0.05 and **P<0.01 as compared to non-irradiated control cells (Mean±SD). n=3

CHAPTER 5
DISCUSSION AND CONCLUSION

5.1 Overview

The aim of this project was to determine whether exposing cells to PBM using laser light with a fluence of 5 J/cm^2 and wavelength of 660 nm and treating them with a low potency homeopathic solution containing *C. officinalis* 3 cH, *H. perforatum* 3 cH and *E. purpurea* 3 cH increased wound healing capacity in diabetic wounded cell models.

Chronic wounds can occur in many disease conditions, including in DM, where non-healing wounds are called DFUs. DFUs are a serious complication in DM as it may lead to lower limb amputations (Sohn *et al.,* 2010). The aetiology of DFUs is multifactorial and may occur due to pathological changes such as ischaemia and neuropathy and altered immune responses present in DM (Tsourdi *et al.,* 2013). DFUs are a burden on healthcare systems, and result in social problems such as disability and decreased quality of life (Ragnarson Tennvall & Apelqvist, 2004; Barg *et al.,* 2017).

Chronic wounds do not follow the regular sequence of wound healing which occurs in a limited timeframe and constitutes the phases of haemostasis, inflammation, proliferation and remodelling (Tsourdi *et al.,* 2013). Chronic wounds are defined as barrier defects that fail to heal in three months (Nunan *et al.,* 2014). They have several cellular and molecular changes which distinguishes them from acute wounds. Among other changes, they exhibit persistent inflammation, possibly infection, hyperproliferative epidermis but stalled re-epithelialisation, sub-optimal granulation tissue, fibroblast senescence, diminished angiogenesis and elevated MMPs (Nunan *et al.,* 2014).

PBM is a healing modality with established wound healing effects, including decreasing pain and inflammation and accelerating healing (Avci *et al.,* 2013; Ramos *et al.,* 2019). PBM is desirable as a therapeutic intervention because it has virtually no adverse effects, is non-invasive and is easy to administer (Avci *et al.,* 2013). PBM refers to the use of red and NIR to stimulate healing. The main chromophores responsible for its beneficial effects are COX, which forms part of the mitochondrial electron transport chain, and calcium ion channels (Hamblin, 2017). The effects of light absorption by these chromophores are increased ATP, a short-lived burst of ROS, increased release of NO and changes in

calcium levels. Tertiary effects are varied and include activation of transcription factors which result in better cell survival, enhanced proliferation and migration and synthesis of new proteins (Hamblin, 2017). Its role in ROS modulation and inflammation seems to be a key player in stimulating healing. In normal cells, PBM increases ROS, however in oxidatively stressed cells or disease models it has the opposite effect, and it can decrease oxidative stress and up-regulate antioxidant defence mechanisms (Hamblin, 2017). Although its mechanism of action is not yet clearly understood, PBM has been shown to stimulate a variety of cellular responses and cause changes in various cell types. PBM acts on fibroblasts, where it increases the expression of genes involved in ECM production (Ayuk *et al.,* 2014) and promotes the remodelling phase of wound healing by stimulating ECM synthesis and decreasing matrix breakdown (Ayuk *et al.,* 2018).

PBM has been the focus of much research for its applications in the medical field. Its efficacy has been investigated for medical conditions as disparate as dermatological conditions (Barolet, 2018), oral mucositis (Pinheiro *et al.,* 2019) skeletal muscle recovery (Fisher *et al.,* 2019) and chronic obstructive pulmonary disease (Miranda *et al.,* 2019). It is favoured as an intervention to treat skin wounds, where it actively promotes healing, probably through its ability to stimulate various physiological processes such as inflammation resolution, neo-angiogenesis, proliferation of epithelial cells and fibroblasts, collagen synthesis and deposition, revascularisation and wound contraction (Andrade *et al.,* 2014). In the context of DFUs, which are notoriously difficult to heal, it effectively stimulates healing as has been demonstrated in human and animal studies (Kaviani *et al.,* 2011; Asghari *et al.,* 2017*;* Yoshimura *et al.,* 2016).

Homeopathy is an alternative health modality that is based on the concept of ‘like cures like’ and has been proven to be effective in the treatment of a variety of health conditions such as upper respiratory tract infections and allergies (Bellavite *et al.,* 2006) and childhood diarrhoea (Jacobs *et al.,* 2003). It is regarded as safer than conventional pharmaceutical drugs (Kirby, 2002) and forms part of publicly funded healthcare systems in various countries such as Austria and France (Relton *et al.,* 2017) and is especially popular in India where it is regarded as part of mainstream medicine (Shrivastava *et al.,* 2015). There is much controversy surrounding homeopathy, not least due to the concept of ‘ultramolecular dilutions’ which refers to the homeopathic practice of serially diluting the material starting substance, in some cases beyond Avogadro’s number (Fisher,

2012). Thus, dilutions beyond 23 X/D or 12 c/cH are very unlikely to contain a molecule of the starting substance (Fisher, 2012). Nevertheless, despite the fact that the mechanisms of action of homeopathy are still unclear, a number of meta-analyses and systematic reviews have been published which proves that homeopathy has curative effects which are not placebo (Linde *et al.,* 1997; Mathie, 2003; Jonas *et al.,* 2003).

In this study, a combination of three low potency (i.e. highly concentrated) homeopathic remedies were tested as a therapeutic intervention. The three remedies were *C. officinalis* 3 cH, *H. perforatum* 3 cH and *E. purpurea* 3 cH. Each of these substances, by themselves, have proven effective in accelerating wound healing in a herbal form (Parente *et al.,* 2012; Samadi *et al.,* 2010; Rezaie *et al.,* 2013). There is very little published data on homeopathic medicines. Homeopathic literature, used in the practice of homeopathy, is based on empirical evidence gathered through homeopathic pathogenic trials, toxicology and clinical experience (Fisher, 2012). This literature indicates all three these remedies as useful in wound healing (Hering, 1997; Boericke, 1999; Sinha, 1981). In addition to this, Bresler *et al.* (2007) found encouraging cellular results in an *in vitro* experiment using homeopathic *C. officinalis* in a 3 cH potency, despite not finding statistically significant differences; *H. perforatum* in a 30 cH potency has also been shown to stimulate nerve regeneration in rats (Mohammadi *et al.,* 2012).

5.2 Effect of 5% Ethanol

The 5% alcohol solvent used in this study caused accelerated wound healing as determined by qualitatively assessing wound closure via microscopy. In addition, cell viability measures (Trypan Blue and ATP luminescence) did not show any harmful effects of the 5% EtOH when compared to the dH2O group. When cytotoxicity was measured, however, there was a significant increase in LDH release showing that cells were more stressed when treated with 2.5%, 5% and 10% EtOH compared to dH2O. Taking all these results into consideration however, the 5% EtOH was chosen as a solvent due to its overall apparently beneficial effect. It would seem from the differences in the morphological changes in wounds and the LDH results that stressing cells does not necessarily equate with decreased migration abilities, and indeed 5% had the opposite effect when looking at the images – it augmented wound healing.

In later experiments, an alcohol control group was included to determine any possible harmful effects of the solvent. Wounded cells treated with medium with 5% EtOH added were compared to their control, which was wounded cells treated with normal medium without 5% EtOH. This is different to the control in the preliminary experiment where the same amount of dH2O was added to serve as a control.

Cells treated with 5% EtOH had increased IL-6 expression compared to the NW control. They also had decreased ATP levels and increased LDH, indicating that the alcohol decreased viability and increased cytotoxicity. In contrast to this, the EtOH caused decreased TNF-α concentration, which is unexpected. Both TNF-α and IL-6 are pro-inflammatory cytokines which are produced by fibroblasts (Brem *et al.,* 2001) and are up-regulated during the inflammatory phase of wound healing (Barrientos *et al.,* 2008). IL-6 is usually detectable immediately after injury, with peak levels reported soon after - within 24 h (Gebhard *et al.,* 2000), while TNF- α levels increase within 1-2 h after injury (Grellner *et al.,* 2000) and reach maximum levels at 84 h after injury (Hirschl *et al.,* 1996).

It remains unclear whether an early increase or decrease in inflammatory markers is desirable. Healing is stimulated when levels of IL-6 and TNF-α (to a limited extent) are increased shortly after injury (Barrientos *et al.,* 2008). However, some authors pronounce that inflammation is a double-edged sword and could be the major cause of scarring (Martin & Nunan, 2015). The timeline of an increase in inflammation is likely a crucial consideration – early inflammation is necessary for healing while persistent inflammation is a hallmark of chronic wounds (Loots *et al.,* 1998). This study investigated changes 48 h after injury and so falls within this early period. It is uncertain to what extent this model is indicative of the chronic wounds of DM, DFUs, however as those tissues have been exposed to an inflammatory milieu for greater than 3 months (Nunan *et al.,* 2014).

What was observed was increased wound closure due to alcohol exposure – an example of a stress condition. It is likely that sub-lethal levels of alcohol may cause cellular stress which could stimulate healing. Singh *et al.* (2016) investigated the effects of sterile inflammation, which could also be seen as a stress condition, induced by endotoxin application in rat wounds. They found that re-epithelialisation and wound closure were significantly increased in endotoxin-treated wounds compared to wounds treated with inflammatory mediator inhibitors. This response mirrors the response found in this study

when adding different concentrations of low concentration alcohol to culture medium. In addition, cell types react differently to alcohol. Alcohol concentrations as low as 1.5% are cytotoxic to human hepatoma cells (Fukaya *et al.*,1997), while others found indications of damage only at a concentration of 8% ethanol after 3 h exposure in an experiment using 4-12% ethanol in rat gastric epithelial cells (Hiraishi *et al.,* 1999), and that the amount of damage was dependent on the concentration and ethanol exposure time.

5.3 Normal Wounded Models

5.3.1 Effect of homeopathic solution in normal wounded cells

No other studies were found which used the specific combination of homeopathic remedies used in this study, limiting comparison to the literature. The homeopathic solution was effective in improving cellular migration when the morphological data is considered – more cells were present in the wounded area at 48 h in homeopathic groups. This effect may be attributed to the 5% alcohol in the solution, as previously discussed. This is supported by the findings of others; Fronza *et al.* (2009) found that treating cells with *C. officinalis* herbal extract increased cellular migration using the scratch assay. Hostanska *et al.* (2012) also used a combination homeopathic complex for investigating wound healing and found that a solution containing *C. officinalis* 4x and *H. perforatum* 4x among other remedies increased cellular migration without affecting proliferation. Bresler *et al.* (2007) found considerable migration of fibroblasts into the central scratch after treatment with *C. officinalis* 3 cH.

Homeopathic solution increased cellular viability as indicated by ATP luminescence; a result also found by Bresler *et al.* (2007) using *C. officinalis* 3 cH. Cytotoxicity was increased as indicated by increased LDH levels. This effect might be due to the effect of the 5% alcohol solvent, however, Bresler *et al.* (2007) found the opposite effect with 3 cH *C. officinalis* also made up in 5% EtOH. There was a slight methodological difference in the study of Bresler *et al.* (2007) in that they added 10 µL of homeopathic solution to 2 mL of culture medium on day 1 and day 2 of the experiment, and the LDH assay was only performed at day 3; while in this study 30 µL of solution (containing *H. perforatum* and *E. purpurea* which Bresler *et al.* (2007) didn't include) was added 30 min after wounding in a single dose, and the LDH assay was performed at day 2.

The homeopathic solution increased IL-6 concentration, indicating a pro-inflammatory effect, however this may be due to the effect of the alcohol solvent. The solution decreased TNF-α concentration, which could indicate suppression of inflammation. Another possibility is that the 48 h timepoint used in this study may just be too early to detect TNF-α levels as they typically peak much later than IL-6 concentration (Hirschl *et al.,* 1996). This study might have missed an 'early peak' of TNF-α as it has been reported that levels increase in the first hours after injury, and then reappear only after days (Grellner, 2002). For this reason, IL-6 results might be a more reliable indicator of inflammation in this study.

5.3.2 Effect of PBM in normal wounded cells

To generate a model of PBM, cells were exposed to 5 J/cm^2 laser light at 660 nm. Irradiation was very effective in accelerating wound healing as indicated by morphological data. Qualitatively assessing the images reveal that more cells migrated into the wounded zone of irradiated cells than control cells and more than in cells treated with homeopathic solution. Others have also found an increase in cellular migration following PBM in wound healing studies (Tricarico *et al.,* 2018; Bresler *et al.,* 2007).

Cellular viability as indicated by ATP luminescence was decreased by laser treatment. The findings of Bresler *et al.* (2007) contradict this – they found irradiation increased ATP levels, although this change was not statistically significant. Laser treatment was not toxic to cells as LDH levels remained unchanged compared to controls. This contradicts what Bresler *et al.* (2007) found – in that study fibroblasts that were irradiated had the highest LDH levels, however the increase did not reach statistical significance.

IL-6 was decreased in irradiated normal wounded cells, displaying an anti-inflammatory response, which corroborates the established anti-inflammatory effects of PBM (Lopes-Martins *et al.,* 2005). TNF-α levels did not change significantly. Lima *et al.* (2014) found that PBM decreased IL-6 concentration 6 h after a surgery intervention in rats, however they did not find a change in TNF-α levels at this timepoint, which supports this study's findings at 48 h.

5.3.3 Effect of homeopathic solution combined with PBM in normal wounded cells

Combination treatment in this study involved irradiating cells that had the homeopathic complex remedy added to culture medium. This treatment was successful in accelerating wound closure as determined by morphological data. In fact, the change was more than in either homeopathically or laser treated cells by themselves, pointing to a synergistic effect. Bresler *et al.* (2007) found that first irradiating cells, then treating them with *C. officinalis* 3 cH induced haptotaxis at the wound margin, but that minimal migration took place when cells were first treated homeopathically, then irradiated. In this case the cells did not seem to be adversely affected by the order in which the remedy was added, and in fact the combination therapy seems to be very favourable if one considers the morphological data, which contradicts Bresler *et al.*'s (2007) findings.

However, the situation is different if one considers the viability data. Combination therapy decreased cellular viability as indicated by Trypan Blue and ATP results, supporting the unfavourable effect of irradiation and homeopathic *C. officinalis* found by Bresler *et al.* (2007). Combination treatment decreased LDH levels indicating that it decreased toxicity, while Bresler *et al.* (2007) found that *C. officinalis* treatment followed by PBM increased cytotoxicity.

The LDH data is in line with what was observed qualitatively in the images, and further supports the notion that combination therapy decreases membrane damage in normal wounded models. Combination treatment increased IL-6 concentration, pointing to a pro-inflammatory effect. Once again this is probably due to the effect of the 5% EtOH solvent of the remedy. Indeed, the effect of irradiation ameliorated IL-6 release in the combination group when it is compared to the homeopathic solution-only group. TNF-α levels were unchanged with this treatment protocol.

5.4 Diabetic Wounded Models

5.4.1 Effect of homeopathic remedy complex in diabetic wounded cells

Diabetic models were consistently exposed to high-glucose medium. There was a marked difference in wound morphology at 48 h in diabetic wounded cells compared to normal wounded cells, which confirms what is known – hyperglycaemia delays wound healing (Markuson *et al.,* 2009), including by inhibiting fibroblast migration (Xuan *et al.*, 2014).

Treatment with the homeopathic complex remedy improved fibroblast migration. This is supported by other literature, in the case of *C. officinalis* (Fronza *et al.,* 2009); however, that study was done on a non-diabetic model, and they used a herbal extract of *C. officinalis*. The wound healing effects of *C. officinalis* in the context of DM have been proven, however in a human trial (Carvalho *et al.,* 2016).

Cellular viability as indicated by Trypan Blue and ATP results was not affected by homeopathic remedy treatment, a favourable outcome, and in line with what Bresler *et al*. (2007) found, however they used non-diabetic cells. In addition, homeopathic solution caused decreased cytotoxicity in diabetic wounded cells as indicated by LDH levels. These results are somewhat different to the findings in the normal and normal wounded groups treated with the homeopathic remedy, where the remedy caused increased cytotoxicity.

Both pro-inflammatory cytokines remained unchanged with homeopathic solution. However, IL-6 concentration of homeopathically treated cells were significantly higher when compared to irradiated models, which is also the trend in the normal and normal wounded models and may possibly be ascribed to the effect of the alcohol solvent as discussed above.

5.4.2 Effect of PBM in diabetic wounded cells

PBM improved cellular migration slightly in diabetic wounded models. This finding is supported by Byrnes *et al.* (2004) who used 4 J/cm^2, 623 nm laser light in wounded diabetic rats, and by Houreld and Abrahamse (2007) in an *in vitro* study using diabetic wounded cells and the same laser parameters as in this study. They found that PBM had a significant effect in improving wound healing as assessed by cellular morphology studies. Kaviani *et al.* (2011) also found that PBM treatment accelerated wound healing in patients with DFUs.

Diabetic wounded irradiated cells had unchanged cellular viability measures. Others have found that PBM increases cellular viability, so this study's results contradict those finding (Esmaeelinejad *et al*. 2014; Jere *et al.*, 2018). Cytotoxicity was increased by irradiation as indicated by elevated LDH levels. This is a surprising finding and contradicts what was

found in the normal models and normal wounded models. Others have found that PBM is not cytotoxic in diabetic wounded cells (Houreld & Abrahamse, 2007).

PBM was found to decrease IL-6 concentration in this model. This supports the established anti-inflammatory effect of PBM (Hamblin, 2017), and Yoshimura *et al.* (2016) have demonstrated that PBM (5.7 J/cm^2, 842 nm) significantly decreases inflammatory cell infiltrates in adipose tissue of hyperglycaemic mice. TNF-α levels remained unchanged, as with most of the interventions in this study.

5.4.3 Effect of homeopathic remedy complex combined with PBM in diabetic wounded cells

Combination therapy increased cellular migration in diabetic wounded cells. Furthermore, this effect was greater than either condition alone, pointing to a synergistic effect. This finding supports the results of Carvalho *et al.* (2016) who found that combining *C. officinalis* oil and PBM (4 J/cm^2, 658 nm) accelerated healing of DFUs in study participants. This effect was also found in normal wounded cells.

Trypan Blue levels show decreased viability; however, ATP levels were increased. These results show contradictory effects with regards to cellular viability. However, there were large error margins with the Trypan Blue results, and much smaller ones with ATP results, thus the ATP results might be more reliable. Another consideration is that, despite the fact that both these assays are used as cellular viability indices, they work on different mechanisms. ATP levels might be increased in cells that are more metabolically active or decreased in cells that are less metabolically active but still viable. The literature shows that PBM increases cellular viability in diabetic models (Esmaeelinejad *et al*., 2014; Jere *et al.*, 2018) and that homeopathic *C. officinalis* combined with PBM increased ATP levels in non-diabetic fibroblasts (Bresler *et al.,* 2007). There is a lack of studies in DM models with the use of the specific combination of *C. officinalis, H. perforatum* and *E. purpurea* as well as combined with PBM.

Combination therapy decreased LDH levels, which was also the effect observed in normal and normal wounded models and is an encouraging result. There was no change in IL-6 concentration with combination treatment compared to controls, however IL-6 concentration were increased to a greater extent in the combination group when

compared to the homeopathic and PBM single treatment protocols. This might be an indication of a greater degree of cellular damage inflicted by these therapies in combination. Houreld and Abrahamse (2007) found that irradiating cells with either 5 J/cm^2 or 16 J/cm^2 increased apoptosis in diabetic and normal models when compared to non-irradiated controls, which indicated that some degree of damage is inflicted by irradiation at least.

The results of the alcohol control group showed increased IL-6 release compared to controls, which could be an explanation for the increased IL-6 seen in the combination therapy group. There was an increase in TNF-α. This contradicts what Lima *et al.* (2014) found, as mentioned above which was unchanged TNF-α levels 6 h after surgery. However, that study evaluated TNF-α levels at 48 h and was performed on diabetic cells.

5.5 Conclusion and Future Recommendations

This study revealed a number of interesting findings related to the combined application of PBM and a 3 cH homeopathic solution for the development of potential treatment of states associated with diabetic injury, such as DFUs.

The wound-healing effects of PBM in the context of DM were confirmed in this study. Irradiating cells greatly enhanced cellular migration into the wound area in normal wounded cells and moderately in diabetic wounded cells, but still more than control cells. However, despite this PBM was found to increase cytotoxicity. It was found to be anti-inflammatory based on IL-6 results.

The alcohol model was noted to have had an independent effect on various cellular parameters. As such, homeopathically-prepared *C. officinalis, H. perforatum* and *E. purpurea* 3 cH improved cellular migration in all models, although to a lesser extent in normal wounded models than PBM. Additional investigation is required as to the role of alcohol with respect to cellular migration. Homeopathic solution decreased cytotoxicity but was not anti-inflammatory based on cytokine responses.

Combination therapy involving homeopathic solution followed by irradiation increased wound healing as evidenced by increased cellular migration. In addition, a synergistic effect was found in both diabetic wounded and normal wounded models in combining the

two therapies. Combination therapy also decreased cytotoxicity and increased cellular viability as indicated by ATP production.

The model used in this study proved its usefulness as a mimic of wounds in DM due to the dysfunctional healing demonstrated by slowed fibroblast migration in diabetic wounded models. In addition, IL-6 release was significantly increased in normal wounded models compared to non-wounded models (data not shown), confirming a pro-inflammatory environment induced by the scratch assay, and making it useful for serving as an example of real-life wounds. The IL-6 and TNF-α results generated in this study indicate the complicated nature of cytokine responses. TNF-α was not altered for most conditions and further investigation to determine effects on cytokine release at various timepoints after wounding is recommended to establish effects over a period of time. It is uncertain whether this model, which investigated effects in wounds over 48 h would be representative of the uniquely deranged cytokine environment present in DFUs.

The alcohol model had an independent effect on various cellular parameters. It would be advisable to prepare homeopathic remedies in distilled water in future *in vitro* studies.

In conclusion, the combination of PBM and a homeopathic complex containing 3 cH *C. officinalis, H. perforatum* and *E. purpurea* improved wound healing in an *in vitro* model of DFUs. More research including further investigation of the role of alcohol preparations and their comparison to distilled water preparations is necessary to confirm these results and to establish the mechanisms of action related to the beneficial effects of the combined treatment, other potencies of the remedies used as well as mother tinctures. This may, in turn, lead to use of optimal treatment combinations for use in *in vivo* models.

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APPENDICES

APPENDIX A

MANUFACTURING METHOD FOR HOMEOPATHIC REMEDY

As performed by Fusion Homeopathics CC, South Africa

All three remedies were manufactured according to the German Homeopathic Pharmacopoeia (GHP/HAB) method 3A

1. Mother tincture* of *C. officinalis*, *H. perforatum* and *E. purpurea* was sourced from reputable Swiss and German suppliers (quality guaranteed by Certificate of Analysis). *The mother tincture consists of the fresh plant extract made by mixing 33.3 parts of the plant to 66.7 parts ethanol to give a final ethanol concentration of 60% (m/m)
2. The first centesimal dilution (1 cH) of the three separate remedies - *C. officinalis*, *H. perforatum* and *E. purpurea* was made by adding 3 parts tincture to 97 parts ethanol (62%) and succussing the solution (vigorously mixing by striking against a firm surface) 100 times
3. The second centesimal dilution (2 cH) of all three remedies were made using 1 part of the 1 cH solution and adding it to 99 parts ethanol (43%) and succussing 100 times
4. The third centesimal dilution (3 cH) was made in combination, by adding each remedy in a ratio of 1:100 to the vehicle, and then succussing the mixture together 100 times. The final required solvent (i.e. distilled water, 2.5%, 5% or 10% ethanol) was used as vehicle in each case.

APPENDIX B

HDC CLEARANCE LETTER

FACULTY OF HEALTH SCIENCES

HIGHER DEGREES COMMITTEE

HDC-23-11- 2019

23 April 2019

TO WHOM IT MAY CONCERN:

STUDENT: **WURZ, J**
STUDENT NUMBER: **216091057**

TITLE OF RESEARCH PROJECT: The Wound Healing Effects of a 3 cH Homeopathic Solution of Hypericum Perforatum, Calendula Officinails and Echinacaea Purpurea Combined with Photobiomodulation on Diabetic Fibroblasts

DEPARTMENT OR PROGRAMME: HOMOEOPATHY

SUPERVISOR: Dr J Pellow **CO-SUPERVISOR:** Prof N Houreld

The Faculty Higher Degrees Committee has scrutinised your research proposal and concluded that it complies with the approved research standards of the Faculty of Health Sciences; University of Johannesburg.

The HDC would like to extend their best wishes to you with your postgraduate studies

Yours sincerely,

Prof S Nalla
Chair: Faculty of Health Sciences HDC
Tel: 011 559 6258
Email: shahcdn@uj.ac.za

APPENDIX C

ETHICS CLEARANCE LETTER

FACULTY OF HEALTH SCIENCES
RESEARCH ETHICS COMMITTEE

NHREC Registration: REC 241112-035

ETHICAL CLEARANCE LETTER
(RECX 2.1)

Student/Researcher Name	Wurz, J	Student Number	216091057
Supervisor Name	Dr J Pellow	Co-Supervisor Name	Prof N Houreld
Department	Homoeopathy		
Qualification	368		
Research Title	The Wound Healing Effects of a 3 cH Homeopathic Solution of Hypericum Perforatum, Calendula Officinalis and Echinacaea Purpurea Combined with Photobiomodulation on Diabetic Fibroblasts		
Date	16 May 2019	Clearance Number	**REC-01-19-2019**

Approval of the research proposal with details given above is granted, subject to any conditions under 1 below, and is valid until 16 May 2020.

1. **Conditions*:**
None

2. **Renewal:**
It is required that this ethical clearance is renewed annually, within two weeks of the date indicated above. Renewal must be done using the Ethical Clearance Renewal Form (REC 10.0), to be completed and submitted to the Faculty Administration office. *See Section 12 of the REC Standard Operating Procedures.*

3. **Amendments:**
Any envisaged amendments to the research proposal that has been granted ethical clearance must be submitted to the REC using the Research Proposal Amendment Application Form (REC 8.0) prior to the research being amended. Amendments to research may only be carried out once a new ethical clearance letter is issued. *See Section 13 of the REC Standard Operating Procedures.*

4. **Adverse Events, Deviations or Non-compliance:**
Adverse events, research proposal deviations or non-compliance must be reported within the stipulated time-frames using the Adverse Event Reporting Form (REC 9.0). *See Section 14 of the REC Standard Operating Procedures.*

The REC wishes you all the best for your studies.

Yours sincerely,

Prof. Christopher Stein
Chairperson: REC
Tel: 011 559 6564
Email: cstein@uj.ac.za

RECX 2.1 – Faculty of Health Sciences
Research Ethics Committee

Secretariat: Ms Raihaanah Pieterse
Tel: 011 559 6073 email: rpieterse@uj.ac.za

APPENDIX D

PERMISSION LETTER

UNIVERSITY OF JOHANNESBURG

07 March 2019

To whom it may concern

Dear Sir/Madam

This letter serves to confirm that Ms Jana Wurz (student number 216091057) is hereby granted permission to conduct her masters' project in the Laser Research Centre Laboratories. Her project, entitled "The wound healing effects of a 3 cH homoeopathic solution of *Hypericum perforatum*, *Calendula officinalis* and *Echinacaea purpurea* combined with photobiomodulation on diabetic fibroblasts", is being conducted under the supervision of Dr J. Pellow and co-supervision of myself. Ms Wurz has previous laboratory and biochemistry experience, and will also be assisted and trained in the laboratory by senior students and members of staff.

Sincerely,

Prof Nicolette Houreld

L.R.C. Laser Research Centre

LASER RESEARCH CENTRE
FACULTY OF HEALTH SCIENCES, PO BOX 17011, DOORNFONTEIN 2028, JOHANNESBURG, SOUTH AFRICA
TELEPHONE: +27 (0) 11 559 6833; FACSIMILE: +27 (0) 11 559 6884
EMAIL:nhoureld@uj.ac.za; WEBSITE: http://www.uj.ac.za/lrc

Laser Applications in Health NRF SARChI

APPENDIX E

LIST OF CONSUMABLES AND CALCULATIONS

E1 List of Consumables

PRODUCT	PROVIDER	CATALOGUE NUMBER
1 mL disposable sterile pipettes	BD Biosciences, The Scientific Group	35 7522
2 mL disposable sterile pipettes	Bibby Sterilin Ltd	40102
5 mL disposable sterile pipettes	BD Biosciences, The Scientific Group	BD 357543
10 mL disposable sterile pipettes	Corning, The Scientific Group	4101
75 cm^2 sterile culture flasks (T75)	Corning, The Scientific Group	431464U
175 cm^2 sterile culture flasks (T175)	Corning, The Scientific Group	431466
96-well clear microplates	Greiner Bio-One	655161
96-well white microplates	Greiner Bio-One	655075
3.4 cm diameter tissue culture plates	Corning, The Scientific Group	430165
Absolute ethanol	Sigma-Aldrich	32221
Amphotericin-B	Sigma-Aldrich	A2942
CellTiter-Glo® 3D Cell Viability Assay	Promega	G9682
Corning pipette tips (1-200 µL)	Corning, The Scientific Group	CR 411S
Corning pipette tips (100-1000 µL)	Corning, The Scientific Group	CR 4114S
Cryoprotective vials	Corning, The Scientific Group	430489
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega	G1782
Biofreeze	Biochrom GmbH	F2270
ELISA kit human TNF-α	Elabscience®	E-EL-H0109
ELISA kit human IL-6	Elabscience®	E-EL-H0102
Eppendorf® microtube (500 µL)	Sigma-Aldrich	Z666521
Eppendorf® microtube (1.5 mL)	Sigma-Aldrich	Z666505
Falcon® centrifuge tube (50 mL)	Corning, The Scientific Group	352070
Foetal bovine serum	Gibco®, Life Technologies	10499-044
(D+) Glucose	SaarChem, Muldersdrift, South Africa	267600
Hanks' Balanced Salt Solution + Ca^{2+} and Mg^{2+}	Sigma-Aldrich	H9394
L-glutamine	Sigma-Aldrich	G7513
Minimum Essential Medium	Sigma-Aldrich	M2279
Non-essential amino acid	Sigma-Aldrich	M7145
Penicillin-streptomycin	Sigma-Aldrich	P4333
Sodium pyruvate	Sigma-Aldrich	S8636
Trypan Blue	Sigma-Aldrich	T6146
TrypLE™ Select (1X)	Gibco®, Life Technologies	12563-029

E2 Media, Chemicals and Solutions

E2.1 Preparation of complete culture media

Constituent	Volume in 50 mL
MEM	42 mL
FBS (10%)	5 mL
Penicillin-Streptomycin (1%)	0.5 mL
Amphotericin B (1%)	0.5 mL
2 mM L-Glutamine	1 mL
0.1 mM Non-essential amino acids	0.5 mL
1 mM Sodium pyruvate	0.5 mL

E2.2 Preparation of complete diabetic media

Constituent	Volume in 50 mL
Complete MEM	49.1 mL
1 M stock D-glucose	0.9 mL

E2.3 Preparation of stock 1 M D-glucose

Constituent	To make 100 mL
D-glucose	18 g
Distilled water (Autoclaved at 121 °C)	100 mL

*The stock solution was stored at 4 °C

E2.4 Calculation of number of cells for seeding in 3.4 cm diameter tissue culture plates

A concentration of 6 X 10^5 cells per 3.4 cm plate was required, in a volume of 3 mL culture medium. The formula used to calculate the number of plates that can be seeded from *n* viable cells is shown here:

Number of plates = Total number of viable cells/Total amount of cells required

Volume to be seeded = Volume required/Number of plates

APPENDIX F

ATCC WS1 DATASHEET

ATCC

Product Sheet

WS1 (ATCC® CRL-1502™)

Please read this FIRST

Storage Temp.
liquid nitrogen vapor phase

Biosafety Level
1

Intended Use

This product is intended for research use only. It is not intended for any animal or human therapeutic or diagnostic use.

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003. To make the complete growth medium, add the following components to the base medium: fetal bovine serum to a final concentration of 10%.

Citation of Strain

If use of this culture results in a scientific publication, it should be cited in that manuscript in the following manner: WS1 (ATCC® CRL-1502™)

American Type Culture Collection
PO Box 1549
Manassas, VA 20108 USA
www.atcc.org

800.638.6597 or 703.365.2700
Fax: 703.365.2750
Email: Tech@atcc.org

Or contact your local distributor

Description

Organism: *Homo sapiens*, human
Tissue:
skin
Disease: normal
Cell Type: fibroblast
Age: 12 week gestation
Gender: female
Morphology: fibroblast
Growth Properties: adherent
DNA Profile:
Amelogenin: X
CSF1PO: 10,13
D13S317: 12
D16S539: 10,11
D5S818: 13
D7S820: 9,10
THO1: 8,10
TPOX: 8,9
vWA: 17,18

Batch-Specific Information

Refer to the Certificate of Analysis for batch-specific test results.

SAFETY PRECAUTION

ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.

Unpacking & Storage Instructions

1. Check all containers for leakage or breakage.
2. Remove the frozen cells from the dry ice packaging and immediately place the cells at a temperature below -130°C, preferably in liquid nitrogen vapor, until ready for use.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at -70°C. Storage at -70°C will result in loss of viability.

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).
2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on should be carried out under strict aseptic conditions.
3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium and spin at approximately 125 x g for 5 to 7 minutes. Discard supernatant.
4. Resuspend the cell pellet with the recommended complete medium and dispense into a 25 cm^2 culture flask. It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).
5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination.