Combining selected medicinal plant extracts to improve wound healing of the skin

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DECLARATION BY RESEARCHER

I, Anri Hattingh, hereby declare that this dissertation, titled “Combining selected medicinal plant extracts to improve wound healing of the skin” is my own work. It has not been previously submitted for any other degree or professional qualification at any institution. I declare that the sources made use of in this dissertation have been referenced and acknowledged. I confirm that this research study was submitted to the Turn-it-in software program. A satisfactory report was attained regarding plagiarism.

A. Hattingh

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ABSTRACT

Throughout history plant-based medicines have been used for the treatment and management of wounds and some of these natural remedies are still very popular. *Aloe vera* gel is a well-known medicinal plant widely used in cosmetic and medicinal products. *Bulbine frutescens,* a plant indigenous to South Africa, has been used as a wound healing agent since ancient times and contains phytochemicals with antioxidant activity that contributes to its wound healing effects. Turmeric (*Curcuma longa*) is an Indian spice, which has been used in remedies all over the world for its wound healing effects. Curcumin is the main curcuminoid extracted from turmeric rhizomes. Numerous studies have been done to investigate the wound healing properties of medicinal plants such as *A. vera* gel, *C. longa* extract (curcumin) and *B. frutescens* gel individually. Medicinal plants, namely *A. vera* gel, *C. longa* extract (curcumin) and both *B. frutescens* gel and aqueous extract, were included in this study.

Nuclear magnetic resonance spectroscopy was utilised to chemically characterise and quantify marker molecules present in the *A. vera* gel. An HPLC analytical method was used to analyse the *C. longa* extract (curcumin) raw material's purity against a curcumin reference standard. The nuclear magnetic resonance (both ^{1}H-NMR and ^{13}C-NMR) spectra for *B. frutescens* gel and *B. frutescens* aqueous extract was utilised to obtain the chemical fingerprints of these plant materials.

The wound healing potential of *A. vera* gel, *C. longa* extract (curcumin) and both *B. frutescens* gel and aqueous extract were determined by means of the wound healing scratch assay on HaCaT cell monolayers after 24 h and 48 h treatment periods. *A. vera* gel*, B. frutescens* aqueous extract and *B. frutescens* gel exhibited pronounced wound healing effects when tested individually, while *C. longa* extract (curcumin) showed less favorable results. *C. longa* extract (curcumin) did not exhibit any wound healing properties when tested individually and therefore, it was not included in any combinations for further wound healing studies. *B. frutescens* aqueous extract and *B. frutescens* gel showed significant increases in wound closure and migration rate values when tested individually. The methylthiazol tetrazolium (MTT) and neutral red (NR) cytotoxicity assays were performed prior to wound healing scratch assays at a range of concentrations to evaluate the percentage cell viability of both HaCaT and 84BR cells after 24 h and 48 h exposure to selected plant materials.

The aim of this study was to determine the *in vitro* wound healing potential of binary combinations of the selected plant materials that improved wound healing individually by conducting the wound healing scratch assay on HaCaT cell monolayers after 12 h and 24 h treatment periods. The

combinations consisted of two of the selected plant materials in different concentration combinations of the individually tested treatment concentration and were evaluated to determine if a more enhanced wound healing effect could be observed as compared to their individual performances. The percentage wound closure and migration rate (um^2/h) for all the experimental values of the individually tested plant materials, as well as the binary combinations, were expressed as a ratio to the untreated control group (the untreated control = 1).

All the *B. frutescens* aqueous extract and *B. frutescens* gel combinations demonstrated an increased tendency to enhance wound closure as compared to their individual performances. *A. vera* gel and *B. frutescens* gel exhibited wound healing properties when tested individually, but combinations of these plant materials showed unfavourable results and lowered the wound healing properties of these individual plant materials. Therefore, it can be concluded that *A. vera* gel and *B. frutescens* gel are better used as individual wound healing agents, than in combination with each other. Combinations of *B. frutescens* extract with *A. vera* gel showed excellent wound healing results. Combinations of *B. frutescens* extract (0.25, 0.50 and 0.75 mg/ml) with *A. vera* gel (0.25 mg/ml) showed significant wound healing results and more than a 2-fold increase in wound closure and migration rate was obtained at 24 h. *B. frutescens* extract and *A. vera* gel could potentially be advantageous for wound healing and be useful as a wound healing agent.

Keywords: *Aloe vera*, *Bulbine frutescens*, *Curcuma longa*, curcumin, scratch assay, wound healing

UITTREKSEL

Deur die geskiedenis heen word plant-gebaseerde medisyne gebruik vir die behandeling en versorging van wonde, en sommige van hierdie natuurlike middels is vandag nog steeds baie populêr. *A. vera* gel is 'n bekende medisinale plant wat baie gebruik word in kosmetiese en medisinale produkte. *B. frutescens*, 'n inheemse plant wat in Suid-Afrika voorkom, word sedert antieke tye vir sy wond genesende eienskappe gebruik en bevat fitochemikalieë met anti-oksidant aktiwiteit waaraan die wond-helende effek daarvan toeskryf kan word. Borrie (*C. longa*) is 'n Indiese spesery wat regoor die wêreld in medisinale produkte gebruik word vir sy wond genesende eienskappe. Curcumin is die belangrikste curcuminoïed wat uit borrie-risome onttrek word. Verskeie studies is gedoen om die wond genesende eienskappe van medisinale plante soos *A. vera* gel, *C. longa* ekstrak (curcumin) en *B. frutescens* gel individueel te ondersoek. Medisinale plante, naamlik *A. vera* gel, *C. longa* ekstrak (curcumin) en beide *B. frutescens* gel en waterige ekstrak, is in hierdie studie ingesluit.

Kern magnetiese resonans spektroskopie is gebruik vir die karakterisering en kwantifisering van merker molekules wat in *A. vera* gel voorkom. 'n HPLC-analitiese metode is gebruik om die *C. longa* ekstrak (curcumin) rou materiaal se suiwerheid teenoor 'n curcumin verwysingsstandaard te analiseer. Die kern magnetiese resonansie (beide ^{1}H-NMR en ^{13}C-NMR) spektra vir *B. frutescens* gel en *B. frutescens* waterige ekstrak is gebruik om die chemiese vingerafdrukke van hierdie plantmateriaal te verkry.

Die wondgenesingseffek van *A. vera* gel, *C. longa* ekstrak (curcumin) asook beide *B. frutescens* gel en waterige ekstrak is ondersoek deur middel van ‘n krapwondtsoets op HaCaT-sel monolae na behandelingsperiodes van 24 h en 48 h. *A. vera* gel, *B. frutescens* gel en *B. frutescens* waterige ekstrak het individueel wondgesesingseienskappe getoon, terwyl *C. longa* ekstrak (curcumin) minder gunstige resultate getoon het. *C. longa* ekstrak het geen wondgenesingseienskappe getoon nie en dit is dus nie by enige kombinasie van plantmateriale ingesluit vir verdere wondgenesingstudies nie. *B. frutescens* waterige ekstrak en *B. frutescens* gel het beduidende toenames in die wond toegroei en migrasietempo getoon wanneer dit individueel getoets word. Die MTT- en NR-sitotoksisiteitstoetseis uitgevoer voor die krapwondtoetse vir ‘n reeks konsentrasies om die persentasie sellewensvatbaarheid van beide HaCaT- en 84BR-selle na 24 h en 48 h blootstelling aan die geselekteerde plantmateriaal te evalueer.

Die doel van hierdie studie was om die *in vitro* wondgenesingpostensiaal van binêre kombinasies van die geselekteerde plantmateriaal te bepaal wat die wondgenesing individueel verbeter het

deur die krapwondtoetse op HaCaT-sel monolae uit te voer na 12 h en 24 h behandelingsperiodes. Die kombinasies bestaan uit twee van die geselekteerde plantmateriaal in verskillende konsentrasie kombinasies van die individuele getoetsde behandelingskonsentrasie en is geëvalueer om te bepaal of 'n verbeterde wondgenesingseffek waargeneem kan word in vergelyking met hul individuele resultate. Die persentasie wond toegroei en migrasie snelheid (um^2/h) vir al die eksperimentele waardes van die individueel getoetsde plantmateriale sowel as die binêre kombinasies is uitgedruk as 'n verhouding tot die onbehandelde kontrolegroep (die onbehandelde kontrole = 1).

Al die *B. frutescens* waterige ekstrakte en *B. frutescens* gel kombinasies het 'n verhoogde neiging getoon om wond toegroei te verbeter in vergelyking met hul individuele resultate. *A. vera* gel en *B. frutescens* gel vertoon wondgenesende eienskappe wanneer dit individueel getoets word, maar kombinasies van hierdie plantmateriaal het ongunstige resultate getoon en die wondgenesende eienskappe van hierdie individuele plantmateriale verlaag. Daarom kan die gevolgtrekking gemaak word dat *A. vera* gel en *B. frutescens* gel eerder as individuele wondgenesingsmiddels gebruik moet word, as in kombinasie met mekaar. Kombinasies van *B. frutescens* ekstrak met *A. vera* gel het uitstekende wondgenesingsresultate getoon. Kombinasies van *B. frutescens* ekstrak (0.25, 0.50 en 0.75 mg/ml) met *A. vera* gel (0.25 mg/ml) het statistiesebeteknisvolle wondgenesingsresultate getoon en meer as 'n tweevoudige toename in wond toegroei en migrasietempo is op 24 h verkry. Kombinasies van *B. frutescens* ekstrak en *A. vera* gel kan moontlik voordelig wees vir wondgenesing en nuttig as ‘n wondgenesingsmiddel.

Sleutelbegrippe: *Aloe vera*, *Bulbine frutescens*, *Curcuma longa*, curcumin, krapwondtoets, wondgenesing

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LIST OF ABBREVIATIONS

A

Acemannan: Acetylated glucomannan/aloverose

α-SMA: Alpha smooth muscle

B

84BR: Dermal fibroblast cell line

D

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

D_2O: Deuterium oxide

E

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

e.g.: For example

F

FBS: Foetal bovine serum

FGF: Fibroblast growth factor

H

HaCaT: Human immortalised keratinocyte cell line

HIV: Human Immunodeficiency Virus

HPLC: High-performance liquid chromatography

I

i.e.: In other words

IL-1: Interleukin-1

M

MMP: Matrix metalloproteinases

MMP-9: Matrix metallopeptidase-9

mRNA: Messenger ribonucleic acid

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide or methyl thiazolyl tetrazolium or methylthiazol tetrazolium

N

NF-(κ)B: Nuclear factor kappa-light-chain-enhancer of activated B cells

NMR: Nuclear magnetic resonance

NR: Neutral red

NSAID: Non-steroidal anti-inflammatory drug

P

PBS: Phosphate buffered saline

PDGF: Platelet derived growth factor

T

TGF-β1: Transforming growth factor-β1

TGF-β: Transforming growth factor- β/ (beta CI)

TNF-α: Tumour necrosis factor alpha

U

UV: Ultraviolet

V

VEGF: Vascular endothelial growth factor

CHAPTER 1 INTRODUCTORY CHAPTER

1.1 Introduction

1.1.1 Wounds

A wound may be defined as a disruption or injury of the skin surface and can extend to deeper underlying tissues causing harm to tendons, vessels, muscles, nerves or other primary structures (Velnar *et al.,* 2009:1529; Hashemi *et al.,* 2015:6). Different types of wounds may be classified according to their aetiology, clinical appearance, location and type of injury. Furthermore, wounds can be clinically categorised into open or closed wounds and acute or chronic wounds (Sabale *et al*., 2012:144). Open wounds have visible bleeding and the underlying tissue of the skin is exposed, while with closed wounds, the skin is still intact (Sabale *et al*., 2012:144). Chronic wounds are recurring and healing exceeds 12 weeks, while acute wounds are usually healed completely within 8 to 12 weeks (Hashemi *et al.,* 2015:2).

1.1.2 Wound healing

According to Flanagan (2000:299), wound healing can be described as the complex physiological process by which the body restores the normal structure and function of damaged tissue. This process of wound healing can be divided into four dynamic phases, namely coagulation together with haemostasis, inflammation, proliferation and remodelling (Velnar *et al*., 2009:1528). Injuries to the skin or tissues cause bleeding and in reaction to this injury, coagulation and haemostasis as well as vasoconstriction immediately takes place. This reaction prevents excessive blood loss (Flanagan, 2000:299). Erythema, oedema, localised heat and discomfort or pain are signs and symptoms frequently associated with the inflammatory phase of wound healing (Flanagan, 2000:299). The inflammatory phase of wound healing can be sub-divided into two phases namely an early and a late inflammatory phase. During the early inflammatory phase, neutrophils enter the wound and use phagocytosis to remove foreign particles and bacteria in order to protect the body against infections. During the late inflammatory phase, the process of phagocytosis is continued by macrophages (Velnar *et al*., 2009:1532). Macrophages produce complex proteins, growth factors and prostaglandins that further facilitate wound healing (Flanagan, 2000:299). The proliferative phase can be sub-divided into three specific stages namely the formation of granulation tissue; wound contraction and re-epithelialisation. The fourth phase of wound healing is remodelling (Flanagan, 2000:299), which involves the formation of new epithelium and maturation of scar tissue (Singh *et al.,* 2017:476). During the latter part of this phase, collagen type III is replaced by collagen type I (Wang *et al*., 2017:95). Thereafter, collagen fibres are re-

arranged to enhance the tensile strength of the wound, which is about 80% in comparison to the strength of normal skin (Singh *et al.,* 2017:476).

Re-epithelisation has a pivotal role in the wound healing process. During re-epithelisation, the rate of proliferation and migration of keratinocytes increases to restore the dead and damaged cells in the wounded area, eventually covering the entire wound area (Santoro & Gaudino, 2004:274). The epidermis consists mainly of keratinocytes and acts as a primary defence barrier for the body against external factors (Rousselle *et al.,* 2019:345).

1.1.3 Wound treatment

Various topical antimicrobials are commercially available that can be used to prevent and treat wound infections (Ousey, 2018:108). However, with the overuse and misuse of antibiotics over the years, a drastic rise in antibiotic resistance has been seen, not to mention the slower development of new antibiotics. This antibiotic predicament raises the need for treatment strategies other than the conventional antibiotic treatment (Negut *et al.,* 2018:2). The use of traditional herbal medicines for wound healing has been reconsidered by many health care professionals (Dorai, 2012:419). Herbs and medicinal plants have been used as wound healing agents since ancient times and their use is still highly valued (Sabale *et al*., 2012:145-148).

Aloe vera is the most well-known and mercantile species among all the species of Aloes and is widely used in the food, cosmetic and pharmaceutical industry (Hamman, 2008:1600). The wound healing properties of materials derived from *A. vera* has been reported in numerous studies (Khorasani *et al.* 2009:587; Oryan *et al*., 2010:509; Shahzad & Ahmed, 2013:225; Hashemi *et al*., 2015:3; Fox *et al.,* 2017:7). *Bulbine frutescens* is endemic to South Africa and has been widely used traditionally for the management and treatment of wounds (Pather *et al*., 2011:364). Throughout history, it has been very popular to treat skin related conditions/diseases and injuries such as burns, bruises, scars, rashes, stings and eczema (Phakamani, 2008:166-167). The popular Indian spice, turmeric (originating from the *Curcuma longa* rhizomes), has been used traditionally for the treatment of various diseases. Curcumin is the main curcuminoid present in turmeric that is responsible for its medicinal properties (Maheshwari *et al*., 2006:2081). Numerous studies reported the wound healing properties of curcumin in the different phases of the wound healing process (Sidhu *et al*., 1998:176; Sidhu *et al.,* 1999:372; Panchatcharam *et al.,* 2006:91; Gadekar *et al.,* 2012:229; Ghosh & Gaba, 2013:767; Akbik *et al.,* 2014:2-6; Yen *et al.,* 2018:612).

1.1.4 Models to investigate wound healing

The use of *in vivo* models to investigate the wound healing properties of medicinal plants involves invasive procedures such as inflicting a circular incision on the skin area (Talekar *et al.,* 2017:75; Anitha *et al.,* 2018:169) or inducing chemical and thermal burns (Hwisa *et al.,* 2013:140). Due to the pain and discomfort it causes, this practice has raised serious ethical concerns and the creation of wounds on live animals to test the wound healing effects of compounds is not acceptable anymore. In addition, the use of animal models may lead to ambiguous results, can be time consuming and very expensive. Therefore, monoculture *in vitro* cell models of skin epidermis origin have been used in numerous studies and can specifically be used to study the effects of compounds on re-epithelialisation, which is a vital part of the wound healing process (Vidmar *et al*., 2017:273). Re-epithelialisation happens during the third phase of the wound healing process, when the rate of keratinocyte proliferation and migration increases, eventually covering the whole wound area (Fox *et al*., 2017:6).

The HaCaT cell line is a non-tumorigenic cell line, which was obtained from normal human trunk skin (Fox *et al*., 2017:2) and regularly used in wound healing research. The scratch assay is one of various techniques that can be used to assess cell migration into the wound area in cell culture models, which is an uncomplicated and inexpensive method (Liang *et al*., 2007:329). This method is straightforward and all equipment required is easily accessible in cell culture laboratories (Liang *et al*., 2007:329). Firstly, the cells are cultured on a flat surface to form a confluent monolayer. Thereafter, a 200 µl micropipette-tip is used to scratch through the monolayer of cells to create a gap or opening which imitates the wound area (Liang *et al*., 2007:329). The percentage wound healing and migration rate of the mechanically induced wounds can be determined by capturing images at several time intervals and measuring the wound gaps after treatment with compounds or plant materials and comparing it with the untreated wounds (Liang *et al*., 2007:329).

1.2 Research problem

Many traditional plant-based medicines have been used for the treatment and management of wounds since ancient times. These medicines comprise of phytochemicals that enhance the wound healing process usually due to their anti-inflammatory, antioxidant, cell proliferation and/or antimicrobial properties (Shah & Amini-Nik, 2017:4). However, most of these traditional wound healing medicines have not been found sufficiently effective in order to be developed into registered medicines. An *in vivo* study by Talekar *et al.* (2017:80) showed that a polyherbal formulation consisting of three plant extracts accelerated wound healing profoundly. This provided proof for the concept of combining medicinal plants in order to obtain improved wound healing effects.

Various studies have been done to investigate the wound healing properties of medicinal plants such as *A. vera* gel, *C. longa* extract, *B. frutescens* extract individually. However, combinations of these selected plant materials have not yet been evaluated to determine if more enhanced wound healing effects can be obtained with combinations of these plant materials, compared to each plant individually. The research problem to be solved in this study is therefore to find out if the selected medicinal plant materials provide improved wound healing properties in combination as compared to that of each individual plant material.

1.3 Aim and objectives of study

The aim of this study is to evaluate different combinations of selected medicinal plant materials on wound healing in an *in vitro* cell culture model. Different concentration combinations of the selected plant materials (which showed wound healing properties when tested individually) were therefore tested for improved wound healing effects by means of a scratch assay in cultured HaCaT cell monolayers.

In order to achieve this aim, the specific objectives of the study were:

- To obtain, prepare and chemically characterise the following selected medicinal plant materials: *A. vera* gel, *C. longa* extract (curcumin), *B. frutescens* extract and *B. frutescens* gel.
- To determine the cytotoxicity potential of the selected plant materials (i.e. *A. vera* gel, *C. longa* extract (curcumin), *B. frutescens* extract and *B. frutescens* gel) at a range of concentrations on HaCaT and dermal fibroblast (84BR) cells, by using cytotoxicity assays with neutral red (NR) and methyl tetrazolium dyes (MTT).
- To determine the *in vitro* wound healing potential of three concentrations of each selected plant material (*A. vera* gel, *C. longa* extract (curcumin), *B. frutescens* extract and *B. frutescens* gel) individually by means of the scratch assay on HaCaT cell monolayers and determine the percentage wound closure and migration rate that were expressed as a ratio to the untreated control group after 24 h and 48 h treatment periods.
- To determine the *in vitro* wound healing potential of combinations of the selected plant materials that improved wound healing individually by conducting the wound healing scratch assay on HaCaT cell monolayers and determine the percentage wound closure and migration rate that were expressed as a ratio to the untreated control group after 12 h and 24 h treatment periods. The combinations will consist of two of the selected plant materials in different concentration combinations of the individually tested treatment concentrations.

1.4 Outline of dissertation

This dissertation consists of 5 chapters. Chapter 1 serves as an introductory chapter that includes a brief background, the research problem, aim and specific objectives of this study as well as the chapter outline of the dissertation. Chapter 2 consists of a literature overview, discussing the wound healing process and diverse wound healing agents including allopathic medicine and medicinal plants. Furthermore, both *in vivo* and *in vitro* models to evaluate wound healing as well as cytotoxicity assays are discussed in this chapter. Chapter 3 consists of the materials and methods used in this study. The results and discussions of this study are stipulated in Chapter 4. Concluding remarks, as well as future recommendations are stated in Chapter 5 of the dissertation. The appendices include comprehensive data acquired from the experiments performed (cytotoxicity and scratch assays) and the ethics approval letter.

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CHAPTER 2 LITERATURE STUDY – THE TREATMENT AND HEALING OF SKIN WOUNDS

2.1 Skin wounds

The skin is the largest organ of the human body, which consists of a complex multi-layer membrane that covers a surface area of approximately 1.8 m^2 in a typical adult human (Aulton & Taylor, 2013:677). It comprises of three distinct layers namely the epidermis, the dermis and the subcutaneous tissue (Negut *et al.,* 2018:3). The skin is vital for life and it is responsible for many important physiological functions such as thermoregulation, sensation, synthesis and storage of vitamin D as well as protection from environmental, chemical and physical hazards (Moore *et al.,* 2015:6). It also acts as a primary defense barrier and protects the human body against biological dangers and ultraviolet radiation from the sun (Forslind, 1995:1; Negut *et al.,* 2018:3).

A wound may be defined as damage to or disruption of the skin layers and/or underlying tissues due to injuries caused by chemical, thermal or physical sources (Hashemi *et al*., 2015:2). Wounds may be classified according to symptoms, aetiology, clinical appearance, location and type of injury. Wounds can be clinically categorised into open or closed wounds, depending on the physical nature and appearance of the wound. Open wounds include incised wounds, lacerations, abrasions and puncture wounds, which have visible bleeding and the superficial layer of the skin is broken to expose underlying layers and/or tissues. Closed wounds, on the other hand, include bruises and haematomas that exist while the skin is still intact (Sabale *et al*., 2012:144). Furthermore, wounds can be classified as acute or chronic based on the physiology and duration of the healing process (Sabale *et al*., 2012:144). Acute wounds normally undergo an orderly and timely healing process (Velnar *et al*., 2009:1529), which results in restoration of the anatomical and functional integrity of the skin within 8 to 12 weeks (Hashemi *et al.,* 2015:2). Chronic wounds fail to proceed through the normal wound healing phases in a timely manner (Velnar *et al.,* 2009:1529) and they are also often recurring. The healing process of chronic wounds exceeds a period of 12 weeks (Hashemi *et al.,* 2015:2).

2.2 The wound healing process

Wound healing can be described as the complex physiological process by which the body restores the normal structure and function of the damaged tissue (Flanagan, 2000:299). According to Lee *et al.* (2012:213), wound healing can be defined as the ability of an organism to repair physical injury by reverting back to the normal continuity of the body structure after disruption of tissues occurred. Various factors can influence the wound healing process. Local factors include the presence of microorganisms, the extent of blood supply, mobility and exposure

to ultraviolet light and foreign objects. Systemic factors refer to the age of the patient, vitamin C and zinc deficiencies, the presence of diseases such as diabetes, haematological abnormalities and use of anti-inflammatory medication such as glucocorticoids (Sabale *et al.,* 2012:144-145).

The process of wound healing can be divided into four dynamic phases, which will be discussed in detail below. The wound healing phases comprise of coagulation (together with haemostasis), inflammation, proliferation and remodelling (Velnar *et al*., 2009:1528).

2.2.1 The coagulation phase

Coagulation, together with haemostasis, takes place to prevent immediate excessive bloodloss post-injury and also provides a matrix for invading cells that are essential at later stages of the wound healing process (Velnar *et al*., 2009:1532). Injuries to the skin or underlying tissues can cause bleeding. The immediate reaction to the injury is vasoconstriction, which is aimed at preventing excessive blood loss (Flanagan, 2000:299). Rapidly thereafter, activation of the coagulation cascade occurs, which leads to platelet aggregation and blood clotting. During this process, the platelets come into contact with collagen, followed by the release of clotting factors that results in blood clot formation (Velnar *et al*., 2009:1532). The blood clot is composed of platelets trapped in a fibrin mesh procured from thrombin splits of fibrinogen in conjunction with small amounts of plasma fibronectin, thrombospondin and vitronectin. As the activated platelets degranulate, cytokines and growth factors are released from the blood clot, which initiates the wound healing process (Martin, 1997:75).

2.2.2 The inflammatory phase

The inflammatory phase of wound healing is associated with erythema, oedema, localised heat and discomfort or pain in the wound area (Flanagan, 2000:299). Wound exudate is produced as the permeability of the blood vessels increases as a result of inflammation. Wound exudate is antimicrobial due to the presence of white blood cells and provides the ideal environment for the growth of phagocytic cells. It also contributes to the wound healing process because it contains growth factors, enzymes, nutrients and proteins (Flanagan, 2000:299).

The inflammatory phase can be sub-divided into an early and a late inflammatory phase. During the early inflammatory phase, neutrophils enter the wound. Neutrophils use phagocytosis to remove foreign particles, bacteria and damaged tissue and destroy them by releasing oxygen-derived free radical species and proteolytic enzymes, in order to protect the body against infections (Velnar *et al*., 2009:1532). Although neutrophils initially play a crucial role to prevent wound infection, it has a negative influence on the repair process by destroying normal tissue and therefore, neutrophils must be eliminated. The life span of neutrophils is short in comparison to

that of macrophages and they are mainly removed by apoptosis. The apoptotic cells are then phagocytosed by macrophages to proceed to the following stages in the wound healing process (Koh & DiPietro, 2011:5).

During the late inflammatory phase, monocytes migrate from the blood into the wound and become macrophages, which replace neutrophils as the predominant leucocyte in the wound (Martin, 1997:75). This phase usually occurs 2-3 days post-injury (Velnar *et al*., 2009:1533). Macrophages are drawn into the wounded area by clotting factors, cytokines (platelet derived growth factor (PDGF), transforming growth factor- β (TGF-β), platelet factor IV and leukotriene B_4), decomposition products of elastin and collagen, and complement products (Velnar *et al*., 2009:1533). Cytokines and growth factors are released as soon as the macrophages have been formed (Martin, 1997:75). The process of phagocytosis of pathogens and cell debris is persisted by macrophages (Profyris *et al*., 2012:2). Macrophages play an important role in all phases of wound healing and produce prostaglandins, growth factors and complex proteins to regulate the process of wound healing (Flanagan, 2000:299). Chemokines, cytokines and growth factors (vascular endothelial growth factor (VEGF) and PDGF) are also released by macrophages and play an important role in granulation tissue formation (Kiya & Kubo, 2019:144). Lymphocytes are attracted by interleukin-1 (IL-1), degradation products of immunoglobulin G and complement components and infiltrate the wound area (Velnar *et al*., 2009:1533). Macrophages also attract endothelial cells and fibroblasts for angiogenesis and fibrillogenesis and this initiates the proliferative phase (Lee *et al.,* 2012:213).

2.2.3 The proliferative phase

The proliferative phase involves diverse processes, which occur concurrently (Singh *et al.,* 2017:475). The main purpose of the proliferative phase is the formation of granulation tissue, re-epithelialisation and the restoration of the vascular system (Guerra *et al.,* 2018:3). The diverse processes involved in this phase include angiogenesis, collagen deposition, granulation tissue formation, wound-contraction and re-epithelialisation (Singh *et al.,* 2017:475). Angiogenesis can be defined as the restoration of the vascular system to provide the necessary oxygen and nutrients to the wound area and eliminate metabolic waste (Flanagan, 2000:299). As soon as the platelet plug has formed, platelets release TGF-β, fibroblast growth factor (FGF) and PDGF that trigger angiogenesis (Singh *et al.,* 2017:475). During wound healing, VEGF plays a very important role in angiogenesis and is comprehensively secreted by keratinocytes. Macrophages, fibroblasts and vascular smooth muscle cells also liberate VEGF (Kiya & Kubo, 2019:147). VEGF together with other cytokines are released, in reaction to hypoxia, to activate endothelial cells, which results in neovascularisation and repair of injured blood vessels. Furthermore, neutrophils activate a group of enzymes in hypoxic tissue called matrix metalloproteinases (MMP), which

endorse angiogenesis through releasing VEGF and repairs the extracellular matrix (ECM) (Singh *et al.,* 2017:475).

Growth factors released from the haemostatic clot activate fibroblasts to proliferate and migrate to the wound (Singh *et al.,* 2017:475). Once in the wound, the fibroblasts produce ECM proteins namely hyaluronan, fibronectins and proteoglycans (Velnar *et al.,* 2009:1534), while collagen and fibronectin are synthesised afterwards. Granulation tissue refers to the pink, vascular new wound matrix formed during the early stages of wound healing and primarily consists of collagen (Singh *et al.,* 2017:475). Healthy granulation tissue has a pinkish colour, while granulation tissue with a darker shade may be an indicator of insufficient blood supply or infection (Flanagan, 2000:300). Fibroblasts synthesise collagen, which is an important component to provide tissue reinforcement and strength (Singh *et al.,* 2017:475). Once adequate ECM is deposited in the wound, fibroblasts change to a myofibroblast phenotype and develop pseudopodia, which attaches to the fibronectin and collagen and facilitates wound contraction (Velnar *et al.,* 2009:1534).

Furthermore, growth factors activate keratinocytes to proliferate and permit re-epithelialisation (Guerra *et al.,* 2018:3). Re-epithelialisation occurs towards the end of proliferation and involves the migration and proliferation of keratinocytes to cover the wound with new epithelium (Santoro & Gaudino, 2004:274).

2.2.4 The remodelling phase

The last phase of wound healing is remodelling, also known as the maturation phase and it can take as long as 2 years. This final phase of wound healing involves the formation of new epithelium and maturation of scar tissue (Singh *et al.,* 2017:476). Initially, scar tissue formed in a wound is reddish and slightly elevated. As the scar matures, it becomes paler as the blood flow decreases (Flanagan, 2000:300). The vascularity and metabolic activity also decrease over time (Velnar *et al.,* 2009:1537). During this phase, there is a fine balance between the synthesis of new cells and the apoptosis of existing cells. It involves the breakdown of excessive ECM, along with replacing collagen type III with collagen type I that is more mature (Wang *et al.,* 2018:95). In comparison with intact tissue, collagen fibres regain approximately 50% of its tensile strength within 3 months and a maximum of only 80% of its original strength can be regained/reached (Singh *et al.,* 2017:476). Abnormalities such as keloid and hypertrophic scars may occur during the final stage of wound healing. Keloid scars usually occurs a while after the wound healed and persist to multiply and spread, in contrast to hypertrophic scars that occur immediately and does not intrude the healthy tissue surrounding the wound (Flanagan, 2000:300).

2.3 Wound healing agents

There are a broad variety of products available on the market to treat wounds, including allopathic medicine and products containing plant materials.

2.3.1 Allopathic medicine used for wound healing

Topical antimicrobials can be used to treat and prevent wound infections. Common signs of wound infection include delayed wound healing together with swelling, erythema, localised heat, severe pain or tenderness and purulent discharge (Ousey, 2018:106). Topical antimicrobials can be formulated as ointments that are more occlusive and ideal for dry lesions. They may also be formulated as creams, which are less messy and best for treating moist lesions. Topical antimicrobials can be sub-divided into two main categories namely antibiotics and antiseptics (Lipsky & Hoey, 2009:1543). Various topical antimicrobial products that contain bacitracin, fusidic acid, metronidazole, silver sulfadiazine and mupirocin are commercially available to prevent and treat wound infections. Some antibiotics target specific pathogens, while others have activity against a broad-spectrum of microorganisms (Ousey, 2018:106). Antibiotic-embedded wound dressings are also available to treat wound infections (Negut *et al*., 2018:2). Although wound dressings provide a moist environment that increases the risk for microbial growth, it also creates the ideal environment to reduce healing time (Negut *et al*., 2018:2). Modern dressings are developed to create a moist environment while preventing infections (Bradshaw, 2011:62). Wound dressings embedded with quinolones, aminoglycosides, tetracyclines and cephalosporins provide protection against wound infection (Negut *et al*., 2018:2).

The prevalence of antibiotic-resistant microorganisms increased over the years due to the overuse and misuse of antibiotics (Negut *et al*., 2018:2). The increase in antimicrobial resistance has become a global threat due to its negative impact on the society, patients and the healthcare system (Friedman *et al.,* 2016:416). In addition, the development of new antibiotics to treat infections has slowed down tremendously. This antibiotic predicament raised the need for treatment strategies other than the conventional antibiotic treatment (Negut *et al*., 2018:2). The use of traditional herbal medicines for wound healing has been reconsidered by many professionals due to the development of multidrug-resistance against allopathic antibiotics (Dorai, 2012:419).

It has been recommended that antiseptics should be used for skin wound treatment instead of antibiotics, considering its wider antimicrobial spectrum and less likelihood to develop resistance (Bigliardi *et al*., 2017:261). Hydrogen peroxide, chlorhexidine, povidone iodine, cadexomer-iodine and silver compounds are a few of the most frequently used antiseptics available. Although

antiseptics such as chlorhexidine, silver nitrate and povidone iodine are effective against a broad spectrum of microorganisms, it is frequently toxic to living tissue (Ousey, 2018:106). Even though hydrogen peroxide is very cheap, it has limited activity and may cause some irritating discomfort to the skin. Chlorhexidine only has a few side effects and has activity against a wide variety of gram-positive, as well as gram-negative bacteria (Lipsky & Hoey, 2009:1544). Povidone iodine is effective against a broad spectrum of microorganisms and no resistance towards it, has been reported yet (Bigliardi *et al*., 2017:18). The use of povidone iodine for wound healing is disputable by virtue of its possible harmful effect on keratinocytes and fibroblasts (Mankowits, 2017:371). In a study conducted by Ghafouri *et al.* (2016:1913), traumatic wounds treated with povidone iodine did not result in a decrease in the rate of infection and showed no beneficial effects. Compared to other iodine products, cadexomer-iodine can possibly cause less damage to tissue (Lipsky & Hoey, 2009:1544). Silver compounds is effective against a broad-spectrum of bacteria due to its various mechanisms of action such as deterioration of cell walls, respiratory enzymes, membranes and ribonucleoproteins (Lipsky & Hoey, 2009:1545).

Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), inhibits cyclooxygenases and thereby reduces prostaglandin synthesis responsible for pain and inflammation. In an *in vivo* study by Da Silva Costa *et al.* (2014:328), topical diclofenac gel treatment resulted in a reduction in the inflammation of skin wounds created on Wistar rats.

2.3.2 Medicinal plants used for wound healing

Various herbs and medicinal plants have been used as wound healing agents throughout history and some of these natural remedies are still popular for the treatment of skin lesions (Sabale *et al*., 2012:145-148). These natural medicines comprise of phytochemicals that enhance the wound healing process mostly due to their anti-inflammatory, antioxidant, cell proliferative as well as antimicrobial properties (Shah & Amini-Nik, 2017:4). However, most traditionally used wound healing medicines have not been found sufficiently effective in order to be developed into registered medicines. An *in vivo* study by Talekar *et al.* (2017:80) showed that a polyherbal formulation consisting of three plant extracts accelerated wound healing profoundly. This provided proof for the concept of combining medicinal plants in order to obtain improved wound healing effects. The wound healing properties of some medicinal plants have been confirmed by means of scientific investigations through different mechanisms of action as described below.

2.3.2.1 Aloe vera

Aloe vera is a succulent plant that belongs to the Xanthorrhoeaceae family (Salehi *et al*., 2018:2). The leaves of this perennial plant contains water storage tissue and can therefore survive in

regions with very low or unpredictable rainfall (Hamman, 2008:1600). A photograph of an *A. vera* plant is shown in Figure 2.1.

Figure 2.1: Photograph of an *A. vera* plant (Silversmith, 2005)

A. vera leaves consist of the external green epidermis, the vascular bundles underneath the epidermis and the inner leaf pulp. The external green epidermis consists mostly of structural components, while the bitter latex excreted by the vascular bundles consists of phenolic compounds (anthraquinones and pre-anthraquinones), anthrones, coumarins, chromones, pyrones and flavonoids (Salehi *et al.,* 2018:3).

A. vera gel is found in the parenchyma cells of the inner leaf pulp and consists principally of water (> 98%) with a variety of polysaccharides. Polysaccharides that have been isolated from *A. vera* pulp include pectin, glucomannan, acemannan, cellulose and hemicellulose (Di Scala *et al*., 2013:52). The inner leaf pulp also contains a broad variety of phytochemicals such as alkaloids, anthrones, anthraquinones, chromones, coumarins, pyrones and flavonoids as well as enzymes, proteins, minerals and vitamins (Salehi *et al*., 2018:3). It is important to note that *A. vera* gel contains a number of active compounds and its biological activity can rather be attributed to a combination of various components than an individual substance (Hamman, 2008:1600). The polysaccharide, acemannan (also known as acetylated glucomannan or aloverose), is an essential functional component of *A. vera* gel that can promote wound healing. It consists of several mannose units positioned within the protoplast of the parenchymatous cells of the inner leaf pulp (Sánchez-Machado *et al*., 2017:96).

An *in vitro* study by Fox *et al*. (2017:7) investigated the wound healing properties as well as the cytotoxic activity of the gel and the whole-leaf materials of three different Aloe species (including *A. vera*, *A. ferox* and *A. marlothii*), which showed favourable results. The Aloe leaf materials

investigated in this study had enhanced wound healing in a scratch assay and showed minimum toxicity towards HaCaT cells when utilising the methyl thiazolyl tetrazolium (MTT) assay. A clinical study was conducted by Shahzad and Ahmed (2013:225) to compare the efficiency of 1% silver sulfadiazine cream and *A. vera* dressing for the treatment of burn wounds. It was found that burn wound healing and pain relief were remarkably faster in patients treated with *A. vera* gel, which is more cost-effective, compared to those treated with 1% silver sulfadiazine. Another clinical study conducted by Khorasani *et al.* (2009:587) showed that the rate of re-epithelialisation and wound healing were quicker in second degree burn wounds treated with *A. vera* cream than those treated with silver sulfadiazine. A synergistic effect was found when *A. vera* and silk fibroin were combined to prepare a nanofibrous scaffold investigated for wound healing and tissue regeneration. This hybrid scaffold also exhibited exemplary physico-chemical properties as well as enhanced fibroblast proliferation (Suganya *et al.,* 2014:135). An *in vivo* study conducted on rats that evaluated the wound healing properties of *A. vera* found that it could induce wound contraction and thereby improve wound healing (Oryan *et al*., 2010:509).

A. vera gel is well-known to be used in cosmetic and medicinal products, which have been claimed to exhibit anti-inflammatory, antifungal, antibacterial as well as wound healing properties (Hashemi *et al*., 2015:3). Additionally, *A. vera* has also been used for treatment of diabetes, detoxification to remove wastes and toxins from the body, the treatment of constipation and gastrointestinal disorders as well as many other immune system deficiencies (Salehi *et al*., 2018:2).

2.3.2.2 Bulbine frutescens

Bulbine frutescens, also known as burn jelly plant, is a perennial plant and belongs to the family of Asphodelaceae (Phakamani, 2008:166). *B. frutescens* grows widespread in Southern Africa and has been used by traditional healers since ancient times (Phakamani, 2008:166). The fleshy leaves of this plant contains a clear gel (Pather *et al.,* 2011:365) and it is traditionally used to treat wounds, burns, itches, cuts and bites. Additionally, root infusions of several *Bulbine* species are used to treat convulsions, diabetes, nausea and vomiting, diarrhoea and rheumatism (Van Wyk, 2011:823). A photograph of a *B. frutescens* plant is shown in Figure 2.2.

Figure 2.2: Photograph of a *Bulbine frutescens* plant (Van Wyk, 2011:819)

Phenylanthraquinones isolated from *B. frutescens* have been shown to exhibit anti-cancer and antiplasmodial activity (Abdissa *et al.,* 2014:67). A study conducted by Bringmann *et al.* (2008:1420) reported that joziknipholones A and B, dimeric phenylanthraquinones isolated from the roots of *B. frutescens*, have activity against the chloroquine resistant strain K1 of *Plasmodium falciparum*, a malaria parasite, as well as moderate activity against murine leukemic lymphoma L5178 cells. The anti-HIV and antioxidant activity of *B. frutescens* was investigated by Shikalepo *et al.* (2018:76) and inhibition of HIV-1 protease and reverse transcriptase as well as good antioxidant activity was found.

Both *B. frutescens* and *B. natalensis* have been widely used traditionally for the management and treatment of wounds (Pather *et al*., 2011:364). An investigation into the effects of these plant species on cutaneous wounds on 12 domestic pigs showed an increase in wound contraction. Both plants caused an increase in wound tensile strength and collagen, protein and DNA content, when compared with untreated wounds (Pather *et al.,* 2011:364). Pather and Kramer (2012:523) performed an *in vivo* study on pigs to specifically investigate the effect of gel from these plants on the differentiation of myofibroblasts, which also indicated an increase in wound contraction.

2.3.2.3 Curcuma longa

Turmeric (*Curcuma longa*) is a plant of which the rhizome is used as an Indian spice and contains polyphenolic compounds called curcuminoids. Turmeric belongs to the ginger family (Zingiberraceae). A photograph of *C. longa* is shown in Figure 2.3. There are three closely related curcuminoids found in turmeric namely curcumin, demethoxycurcumin and

bisdemethoxycurcumin. Owing to these curcuminoids, dried turmeric rhizome powder has a yellow colour (Akram *et al*., 2010:65). Turmeric has been used in medicinal products all over the world because of its anti-inflammatory, antioxidant, antimicrobial, anti-cancer (Maheshwari *et al*., 2006:2082), anti-coagulant, anti-mutagenic, anti-infective, as well as wound healing effects (Akbik *et al.,* 2014:1). Curcumin, the main curcuminoid, is predominantly responsible for these medicinal properties (Maheshwari *et al*., 2006:2082). Throughout history, it has been popular in treating various conditions such as diabetic ulcers, cough, rheumatism, anorexia and sinusitis (Akbik *et al.,* 2014:1). When curcumin is administered orally, it is subjected to extensive hepatic pre-systemic clearance and only traces of the compound are able to reach the systemic circulation (Akbik *et al.,* 2014:6).

Figure 2.3: Photograph of a turmeric (*Curcuma longa*) plant, rhizomes and powder (Sa & Das, 2008:14)

Several studies have been conducted showing that curcumin has an impact on various stages in the multi-faceted process of wound healing (Akbik *et al.,* 2014:2). Curcumin was found to promote wound healing by increasing DNA and total collagen production (Ghosh & Gaba, 2013:767). During the inflammatory phase of wound healing, curcumin topical treatment reduces inflammation. It inhibits the production of interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α), as well as the activity of NF-(κ)B (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription factor (Akbik *et al.,* 2014:6). In a study by Yen *et al.* (2018:612), topically applied curcumin was found to enhance wound healing by increasing TNF-α mRNA and protein expression, followed by a significant downregulation in TNF-α expression. It also resulted in an

increase in collagen production (Yen *et al.,* 2018:612). Curcumin was found to reduce matrix metallopeptidase-9 (MMP-9) production and increased alpha smooth muscle (α-SMA) expression through inhibition of NF-(κ)B activity in fibroblasts treated with TNF-α (Yen *et al.,* 2018:605). In a recent study by He *et al.* (2018:325), curcumin/polyvinyl alcohol hydrogel was found to exhibit antibacterial activity and also accelerated wound healing.

In a study by Gadekar *et al.* (2012:229), the wound healing potential of curcumin-containing transdermal patches was investigated. The patches were applied to excision wounds on rats, which resulted in faster wound contraction and healing. Furthermore, the antioxidant potential of curcumin was investigated and conspicuous inhibition of induced damage to fibroblasts and keratinocytes were found. Compared to untreated wounds, transforming growth factor-β1 (TGF-β1) proteins are higher in curcumin-treated wounds leading to faster re-epithelialisation (Sidhu *et al.,* 1998:177). Curcumin-treated diabetic wounds also showed a meaningful increase in the expression of TGF-β1. This may lead to earlier re-epithelialisation together with accelerated tissue integrity renewal (Sidhu *et al.,* 1999:372). An *in vivo* study conducted on rats and guinea pigs to evaluate the wound healing effects of curcumin, showed that there was faster wound closure and thereby indicated enhanced wound healing (Sidhu *et al.,* 1998:176). Macrophage, neutrophil and fibroblast infiltration were higher in curcumin-treated wounds compared to untreated wounds (Sidhu *et al.,* 1998:176). Compared to untreated wounds, curcumin-treated wounds contained a larger number of myofibroblasts. Fibroblasts differentiate and form myofibroblasts, which play a key role in wound contraction (Sidhu *et al.,* 1998:176). In another study by Sidhu *et al.* (1999:373) a greater number of myofibroblasts were also detected in curcumin-treated diabetic wounds.

Panchatcharam *et al.* (2006:91) topically applied curcumin on full-thickness excision wounds on the back of rats and found an increase in collagen synthesis as well as prior maturation of collagen fibres. Furthermore, it also showed faster wound contraction and an increase in tensile strength and shrinkage temperature (Panchatcharam *et al.,* 2006:92). The antioxidant activity of curcumin to prevent oxidative damage has also been substantiated by the decrease in lipid peroxide and an increase in superoxide dismutase, glutathione peroxidase and catalase (Panchatcharam *et al.,* 2006:93-95).

2.4 Models that can be used to evaluate wound healing effects

2.4.1 *In vivo* models

In vivo models that are utilised for wound healing experiments make use of live animals and involve invasive procedures (Anitha *et al*., 2018:169). In a study by Talekar *et al.* (2017:75) to

determine the potential wound healing effects of polyherbal formulations, a circular incision was made in the skin on the dorsal area of Wistar rats. To evaluate the wound healing properties of different types of honey, Hwisa *et al*. (2013:140) induced chemical and thermal wounds on White New Zealand rabbits by using concentrated hydrochloric acid (HCl 35% *v/v*) and a heated metal rod. In another study by Da Silva Costa *et al.* (2014:328), wounds were intentionally inflicted on Wistar rats utilising a metallic punch to investigate the effects of diclofenac on wound healing. However, due to the pain and discomfort that these types of wound inductions cause the animals, this practice has raised serious ethical concerns. Fortunately, the creation of wounds on live animals to test the wound healing effects of compounds is currently not acceptable and therefore not allowed anymore (Meigs *et al*., 2018:275-300).

The use of alternative methodologies have led to the contentment by the consumers of the wound healing products. In addition, the use of animal models is time consuming and very expensive. Furthermore, the use of alternative testing methods has positive consequences due to a reduction in false negative and false positive results (Meigs *et al*., 2018:300).

2.4.2 *In vitro* models

Cell culture models offer a platform for alternative assays during drug discovery and development (Vidmar *et al*., 2017:269). Various assays on cell culture models are available to evaluate wound healing, which include scratch, proliferation, cell viability, cell invasion, genomic and proteomic assays (Vidmar *et al*., 2017:273). Monoculture cell models of corneal, skin epidermis, kidney epithelial and intestinal epithelial origin have been used in numerous studies to investigate the complex process of wound healing (Vidmar *et al*., 2017:273). Monoculture models can specifically be used to study the effects of compounds on re-epithelialisation, which is a vital part of the wound healing process (Vidmar *et al*., 2017:273). Re-epithelialisation happens during the third phase of the wound healing process, when the rate of keratinocyte proliferation and migration increases, eventually covering the whole wound area (Fox *et al*., 2017:6). The inflammatory response, angiogenesis and tissue repair are all influenced by cell migration, which plays a crucial role in these biological processes as well as wound healing (Ascione *et al.,* 2016:123). When cells migrate, it can occur either as individual cells, agglomerates of loosely attached cells or a layer of cells in a group (Kramer *et al.,* 2013:15).

Skin epidermis cell models such as primary keratinocytes, epidermal stem cells and human immortalised keratinocytes (e.g. HaCaT cell line) are useful to study the complex processes of wound healing. The cells can be grown *in vitro* to form a confluent monolayer of cells on which a wound can be induced and thereafter, a number of assays can be conducted to evaluate the wound healing process. The involvement of growth factors, cytokines and the expression of

extracellular markers have been reported in numerous studies using aforementioned models (Vidmar *et al*., 2017:273). Keratinocytes, fibroblasts and melanocytes are the three main cells that the skin consists of (López-García *et al*., 2014:44).

The scratch assay is an uncomplicated and inexpensive cell culture method that is frequently used to investigate wound healing (Liang *et al*., 2007:329). The wound healing scratch assay is often used to investigate *in vitro* cell migration (Ascione *et al.,* 2016:123) in a variety of cell lines including fibroblasts, keratinocytes, endothelial cells, normal epithelial cells and epithelial/mesenchymal cancer cells (Kramer *et al.,* 2013:15). Disadvantages of this assay, however, includes the difficulty in creating a wound that is always even in size by utilising a manual action with a pipette tip (Kramer *et al.,* 2013:15). Preceding wound closure, the migration rate of cells generally increases. It is for this reason that the wounds should be even in size (Kramer *et al.,* 2013:15). The cells are grown on a flat surface to form a confluent monolayer, whereafter a linear scratch or "wound" is created across the monolayer by pulling a pipette tip across the cell layer (Liang *et al*., 2007:329). The cell migration rate can be determined by taking images at several time intervals after the wound has been created mechanically (Liang *et al*., 2007:329). The reduction in wound size can also be calculated as a function of time. The wound starts to "heal" when the cells progress from the unharmed regions into the scratched area to close the "wound" (Kramer *et al.,* 2013:15). Cell-cell interactions are re-established as the cells on the margin of the mechanically created wound grow and closes the opening (Liang *et al*., 2007:329).

The CytoSelect™ assay kit is an *in vitro* model utilised to investigate wound healing and cell migration. The CytoSelect™ wound healing assay consists of inserts that is placed into the well to prevent the cells from growing in a specific area on the bottom surface of the well. After the cells have formed a monolayer, the inserts are removed leaving behind a perfect "wound" or uniform gap/opening across the bottom surface of the well. As the cells start to proliferate and migrate to close the wound gap, the rate of cell migration and proliferation as well as wound closure can be calculated (Cell Biolabs Inc., 2019:2). The uniformity in wound size of the CytoSelect™ assay is definitely an advantage over the scratch assay. However, this wound healing assay kit is very costly and in experiments where multiple replicates have to be performed, it can be a major drawback (Vidmar *et al*., 2017:277).

2.5 Cytotoxicity assays

The effect of substances on cell growth, reproduction and morphology can be evaluated *in vitro* on cells, which are referred to as cytotoxicity assays (Li *et al*., 2015:617). The discovery and development of new therapeutic agents is a very expensive and time-consuming process. Therefore, preclinical testing is key to identify suitable candidates prior to clinical research trials.

Initial preclinical screening of new therapeutic agents can be performed utilising cell cultures (Van Tonder *et al*., 2015:48).

2.5.1 The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay

In 1983, Mosmann (1983:56) developed the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to specifically evaluate cell viability and proliferation. MTT is a tetrazolium salt that is transformed into insoluble purple formazan crystals by succinate dehydrogenase enzymes in the mitochondria of living cells (Fotakis & Timbrell, 2006:172). As mentioned before, Fox *et al*. (2017:2) used the MTT assay to establish the cytotoxicity of selected Aloe gel and whole-leaf materials on keratinocytes (HaCaT cells). The MTT cytotoxicity assay has certain limitations and can, however, be affected by redox reactions caused by vitamins, phytoestrogens, polyphenols and a few metallic compounds and alloys (Perez *et al*., 2017:43). False-positive results can be obtained as certain plant extracts interfere with the MTT salt resulting in abnormally high cell viability values (Karakaş *et al.,* 2017:921).

2.5.2 The neutral red (NR) assay

The neutral red assay is more sensitive when compared to other cytotoxicity assays (e.g. MTT) (Repetto *et al*., 2008:1125). Neutral red dye permeates the cell membrane and accumulates in the lysosomes of metabolically active cells (Fotakis & Timbrell, 2006:172). Due to its versatility, the neutral red assay has diverse biomedical as well as environmental applications (Repetto *et al*., 2008:1125). However, destruction of the cell surface or membrane caused by test compounds could interfere with the accumulation of neutral red dye in the lysosomes of living cells (Triglia *et al.,* 1991:239).

2.6 Summary

Wound healing is a complex physiological process and involves four overlapping phases namely coagulation, inflammation, proliferation and remodelling (Velnar *et al*., 2009:1528; Wang *et al.,* 2018:94; Singh *et al.,* 2017:473). In short, a wound may be defined as damage of the skin surface and/or underlying tissues due to any injury or cut (Sabale *et al* 2012:143).

Since ancient times, plant-based medicines have been used for the treatment and management of wounds and some of these natural remedies are still very popular (Sabale *et al*., 2012:145-148). *A. vera* gel is a well-known medicinal plant widely used in cosmetic and medicinal products considering it has anti-inflammatory, antifungal, antibacterial as well as wound healing properties (Hashemi *et al*., 2015:3; Hamman, 2008:1608). Numerous studies have been done to investigate the wound healing properties of *A. vera* gel (Khorasani *et al.,* 2009:587; Oryan *et al.,* 2010:509;

Shahzad and Ahmed, 2013:225; Suganya *et al.,* 2014:135; Fox *et al.,* 2017:7). *B. frutescens,* a plant indigenous to South Africa, has been widely used traditionally for the management and treatment of wounds (Pather *et al*., 2011:364). The wound healing potential of *B. frutescens* has been observed in *in vivo* studies conducted on pigs (Pather *et al.,* 2011:364; Pather & Kramer, 2012:523). Curcumin is the main curcuminoid extracted from turmeric (*C. longa*) rhizomes, a well-known Indian Spice (Akram *et al*., 2010:65). Turmeric has been used in remedies all over the world because of its anti-inflammatory, antioxidant, anti-cancer and wound healing effects (Maheshwari *et al*., 2006:2082). The wound healing effects of curcumin has also been observed in numerous studies through various mechanisms of action (Sidhu *et al*., 1998:176; Sidhu *et al.,* 1999:372; Panchatcharam *et al.,* 2006:91; Gadekar *et al.,* 2012:229; Ghosh & Gaba, 2013:767; Akbik *et al.,* 2014:2; Yen *et al.,* 2018:612).

Animal models utilised for wound healing experiments involve the intentional infliction of wounds (Hwisa *et al*., 2013:140; Da Silva Costa *et al.,* 2014:328; Talekar *et al.,* 2017:75), which raises serious ethical concerns due to pain and discomfort (Meigs *et al*., 2018:275-300). Various wound healing assays are available that can be performed on monoculture cell models (Vidmar *et al*., 2017:273). The three main cells that the human skin consists of are keratinocytes, fibroblasts and melanocytes (López-García *et al*., 2014:44). The wound healing scratch assay is a straightforward and inexpensive cell culture method (Liang *et al*., 2007:329). Numerous studies have been conducted utilising this method to evaluate wound healing effects of plant materials (Fox *et al*., 2017:6; Talekar *et al.,* 2017:75; Anitha *et al*., 2018:169).

2.7 References

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CHAPTER 3 MATERIALS AND METHODS

3.1 Materials and reagents used

The dehydrated gel of *A. barbadensis* Miller (*A. vera* (L.) Burm.f.) was procured from Improve USA, Inc. (DeSoto, Tx, USA, certified by the International Aloe Science Council), whereas curcumin, extracted from *C. longa*, was purchased from Chromadex Inc. (CA, USA). Fresh *B. frutescens* leaves were collected by Chris van Niekerk on 20/09/2019 from the Botanical Garden of the North-West University, South Africa. A specimen voucher (accession number 15318) has been deposited in the North-West University's Herbarium. Dried *B. frutescens* plant material were obtained from Botanica Natural Products located in rural Limpopo, South Africa. The HaCaT cells were donated by Prof. Joana Miranda from the Department of Toxicological and Bromatological Sciences, Faculty of Pharmacy, University of Lisbon, Portugal. The 84BR cell culture was purchased from the European Collection of Authenticated Cell Cultures (ECACC, #90011805, Salisbury, UK). The various consumables used for the *in vitro* cell culture experiments, with the suppliers, are listed in Table 3.1.

Table 3.1: Chemical substances used during *in vitro* cell culture studies

Substances	Company/supplier
Phosphate buffered saline (PBS) (1X); Dulbecco's Modified Eagle's Medium (DMEM);	HyClone, Separations, Johannesburg, South Africa
Trypsin-Versene® EDTA (0,25%); non-essential amino acids; penicillin/streptomycin (Pen/Strep) (10 000 U/ml); L-glutamine (200 mM)	Lonza, Whitehead Scientific (Pty) Ltd., Cape Town, South Africa
Trypan blue (0.4%); MTT reagent (Thiazolyl Blue Tetrazolium Bromide salts); dimethyl sulfoxide (DMSO); Triton X-100; Neutral Red Solution (0.33%)	Sigma-Aldrich, Merck KGaA, Johannesburg, South Africa
Foetal bovine serum (FBS)	ThermoFisher Scientific, Johannesburg, South Africa

3.2 Experimental design and layout

In vitro studies have been performed on HaCaT and 84BR cell cultures to investigate the wound healing properties of selected medicinal plant materials (i.e. *A. vera* gel, *C. longa* extract (curcumin), *B. frutescens* aqueous extract *and B. frutescens* gel material). The unique aspect of this study, however, is the investigation of the wound healing properties of combinations of the selected plant materials. Improved wound healing effects with combinations have not yet been evaluated previously to determine if combinations of medicinal herbal extracts could enhance the wound healing effect.

Initially, *A. vera* gel, *C. longa* extract (curcumin), *B. frutescens* aqueous extract and *B. frutescens* gel were each tested individually at a range of concentrations for any cell viability reducing effects (i.e. toxicity) on two selected human skin cell lines (i.e. HaCaT and 84BR cell lines). Both methylthiazol tetrazolium (MTT) and neutral red (NR) cytotoxicity assays were used to assess the effects on cell viability. Three concentrations from each plant material were then tested individually for their wound healing potential by means of the scratch assay. Thereafter, different combinations of the selected plant materials (which showed wound healing properties when tested individually) were tested for improved wound healing effects. The combinations consist of two of the selected plant materials at a time in different concentration combinations of the individually tested treatment concentrations. Table 3.2 provides the various concentration combinations of the plant materials (that showed wound healing properties when tested individually) that were evaluated for improved wound healing effects.

Table 3.2: Combinations of the selected plant materials

AVG:BFE*	AVG:BFG*	BFE:BFG*
1A:1B[#]	*1A:1C*[#]	*1B:1C*[#]
1A:2B[#]	*1A:2C*[#]	*1B:2C*[#]
1A:3B[#]	*1A:3C*[#]	*1B:3C*[#]
2A:1B[#]	*2A:1C*[#]	*2B:1C*[#]
2A:2B[#]	*2A:2C*[#]	*2B:2C*[#]
2A:3B[#]	*2A:3C*[#]	*2B:3C*[#]
3A:1B[#]	*3A:1C*[#]	*3B:1C*[#]
3A:2B[#]	*3A:2C*[#]	*3B:2C*[#]
3A:3B[#]	*3A:3C*[#]	*3B:3C*[#]

* AVG = *A. vera* gel, BFE = *B. frutescens* extract, BFG = *B. frutescens* gel. [#] *A. vera* gel: 1A = 0.25 mg/ml; 2A = 0.50 mg/ml; 3A = 0.75 mg/ml; *B. frutescens* extract: 1B = 0.25 mg/ml; 2B = 0.50 mg/ml; 3B = 0.75 mg/ml; *B. frutescens* gel: 1C = 0.25 mg/ml; 2C = 0.50 mg/ml; 3C = 0.75 mg/ml.

3.3 Preparation of selected plant extracts

3.3.1 Preparation of *Bulbine frutescens* gel material

The method as previously published by Pather and Kramer (2012:524) was adapted to obtain the gel material (inner fleshy part of the leaves) from the fresh *B. frutescens* leaves. In short, the leaves were washed, wiped with 70% ethanol and the fresh gel material was collected from the leaves by removing the rind of the plant material. Thereafter, the fresh gel materials were lyophilised in a freeze dryer (VirTis, UK) and the acquired dried plant material was ground with a Mixer Mill MM 400 (Retsch, Germany) to obtain a fine powder.

3.3.2 Preparation of *Bulbine frutescens* plant extract

An aqueous suspension of dried *B. frutescens* powder in water was prepared at a ratio of 1:5 (plant material: water). This means that for each 20 g of dried plant powder, a volume of 100 ml distilled water was added to prepare the suspension. The suspension was sonicated for 45 min at 45°C in a digital Eumax® ultrasonic cleaner (Labotec, Midrand, South Africa). Subsequently, the suspension was centrifuged at 5 000 x g for 10 min using a Sigma 3-16 KL Laborzentrifugen (Germany). The supernatant was collected and 70 ml of water was added to ensure that all the particles were dissolved. The process was repeated by re-suspending the pellet in 100 ml water, sonicated and centrifuged as described above, followed by the addition of 70 ml water to the supernatant. The combined supernatants were then centrifuged at 1 218 x g for 5 min, followed by filtration through Whatman™ filtration paper with a pore diameter of 125 µm (#1114125, Sigma-Aldrich, Johannesburg, South Africa). The collected filtrate was stored overnight in a -80°C freezer, lyophilised with a VirTis freeze dryer and crushed to obtain a fine powder.

3.3.3 Characterisation of plant materials

3.3.3.1 Characterisation of *Aloe vera* gel

The method as previously described by Jiao *et al.* (2010:843) was utilised for quantification of marker compounds (i.e. aloverose, glucose, lactic acid and malic acid) in *A. vera* gel. An Avance III HD 500 MHz spectrometer (Bruker, Rheinstetten, Germany) was used to record the nuclear magnetic resonance (^{1}H-NMR) spectra of *A. vera* gel. The spectrometer was equipped with an automated sample changer and BBFOPLUS SmartProbe. Nicotinamide was utilised as an internal standard to quantify the amount of marker molecules.

3.3.3.2 Chemical fingerprinting of *Bulbine frutescens* gel and aqueous extract with nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (both ^{1}H-NMR and ^{13}C-NMR) spectra of *B. frutescens* gel and aqueous extract were obtained to chemically fingerprint these plant materials. Approximately 50 mg of both *B. frutescens* gel and aqueous extract were prepared in 2 ml D_2O, respectively. After the solutions were filtered through cotton wool, a small amount of 3-(trimethylsilyl) propionic acid-D4 sodium salt was added, followed by recording of the nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra.

3.3.3.3 Chemical characterisation of *Curcuma longa* extract (curcumin)

The purity of the raw material of *C. longa* extract (curcumin) purchased from Chromadex was analysed against a curcumin reference standard by using a high-performance liquid chromatography (HPLC) method as previously described by Jayaprakasha *et al.* (2002:3669) and Maiti *et al.* (2007:156). Since a validated HPLC analysis method has been used, the method was verified by linearity to establish the correlation between peak area and concentration of curcumin. Approximately 10 mg of both the curcumin reference standard and curcumin extract test sample were dissolved in 10 ml methanol (1 mg/ml), where after 5 ml of each solution was further diluted with an equal amount of methanol (5 ml) to obtain a final stock concentration of 500 µg/ml. A two-fold serial dilution was prepared with methanol between the range of 500 µg/ml to 37.5 µg/ml. The curcumin samples were analysed with an Agilent 1100 HPLC system (Agilent® Technologies, Palo Alto, California, USA) fitted with an autosampler, Quaternary Core pump, diode array ultraviolet (UV) detector, Venusil XBP (C18) column, and Agilent® Technologies OpenLAB CDS ChemStation data acquisition and processing software. An isocratic elution method was used, with 10 µl of sample injected into the mobile phase at a flow rate of 1 ml/min for 8 min. The mobile phase consisted of (A) methanol, (B) 2% acetic acid in water, and (C) acetonitrile at a solvent gradient of 5% (A), 35% (B) and 60% (C). The UV detector was set at a wavelength of 425 nm.

3.3.4 Preparation of the plant material test solutions for cytotoxicity studies

For the cytotoxicity studies on *A. vera* gel, *B. frutescens* aqueous extract and *B. frutescens* gel, test solutions with concentrations ranging from 0.1 to 2.0 mg/ml were investigated, while for *C. longa* extract, test solutions with a concentration range from 1 to 20 µg/ml were investigated (Fox *et al*., 2017:2, Scharstuhl *et al.,* 2009:716). For each of the *A. vera* gel*, B. frutescens* gel and *B. frutescens* aqueous extract, a 2 mg/ml stock solution was prepared in growth medium in a 20 ml volumetric flask. This was followed by diluting each stock solution with the required amount of

growth medium to obtain a range of test solution concentrations, which included concentrations of 0.25; 0.5; 0.75; 1.0; 1.25; 1.5 and 2.0 mg/ml.

The curcumin stock solution was prepared by dissolving 4 mg in 1 ml DMSO, where after 100 µl was further diluted with growth medium to a final volume of 20 ml to obtain a final stock concentration of 20 µg/ml. The DMSO concentration in the test solutions, to which the cells were exposed, was never higher than 0.5% *(v/v)*. A vehicle control group consisting of cells treated with 0.5% DMSO in growth medium was included during the MTT and NR cytotoxicity assays. The curcumin stock solution was diluted with the required amount of growth medium to obtain a range of test solution concentrations, which included concentrations of 1; 2.5; 5; 7.5; 10; 15 and 20 µg/ml.

To prevent contamination of the cells during the cytotoxicity studies, the stock solutions were sterilised before applying the solutions to the cells. For sterilisation of the curcumin stock solution, it was filtered through a 0.22 µm syringe filter (Anatech Instruments (Pty) Ltd., Johannesburg, South Africa). For sterilisation of the *A. vera* gel, *B. frutescens* gel and *B. frutescens* extract stock solutions, which were too viscous to be filtered through a 0.22 µm syringe filter, a pasteurisation technique was applied (Swanepoel, 2018:81). Pasteurisation involved heating the solution to 73°C and maintaining this temperature for 15 s, which was followed by immediate cooling to 9°C in an ice bath. The temperature of the solutions during the pasteurisation technique was monitored with a Digitron thermometer (Digitron, 2088T, Sifam Instruments Limited Torquay, England). To remove undissolved particles, the *A. vera* gel and *B. frutescens* gel solutions were filtered through 0.45 µm syringe filters (Anatech Instruments (Pty) Ltd., Johannesburg, South Africa) after the pasteurisation technique.

3.4 *In vitro* cell culture techniques

3.4.1 Cell culture maintenance

As mentioned before, the cytotoxicity assays were performed on both the human immortalised keratinocyte cell line (HaCaT) and the dermal fibroblast cell line (84BR), while the wound healing scratch assay was only performed on HaCaT cell monolayers.

The HaCaT cells were cultured at 37°C in a 95% humidified environment with 5% CO_2 and maintained in growth medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) (high glucose, 4.5 g/l) supplemented with 10% (*v/v*) foetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine and 1% non-essential amino acids. The HaCaT cells were used between passages 35 and 44 for all the experiments.

The 84BR cell culture was cultivated in growth medium consisting of DMEM (high glucose, 4.5 g/l) supplemented with 15% (*v/v*) foetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine and 1% non-essential amino acids, and incubated at 37˚C in a 95% humidified environment with 5% CO_2. The 84BR cells were used between passages 7 and 12 for the cytotoxicity assays.

The spent growth medium was replaced every second or third day and the cells were inspected regularly for any signs of contamination. The cells were sub-cultured into new flasks or seeded for experiments when they reached approximately 80% confluence. After removing the growth medium, the cells were washed twice with 10 ml PBS to remove all dead cells and remaining growth medium. Thereafter, the cells were treated with 3 ml trypsin-EDTA solution for 9-10 min (HaCaT cells) or 4-5 min (84BR cells) at 37˚C to ensure detachment. After trypsinisation, the flask was agitated with the hand to ensure complete detachment of cells, following the addition of 6 ml of growth medium to neutralise the trypsin. Cell pellets were obtained at 140 *x g* centrifugation for 5 min. The supernatant was discarded and the pellet was re-suspended in 5 ml of fresh growth medium.

Prior to seeding, the number of viable cells within the re-suspended pellet needed to be established to ensure that the cells are seeded according to the correct cell density. The number of HaCaT cells in suspension was determined utilising a haemocytometer with trypan blue exclusion. Firstly, 25 µl trypan blue were mixed with 15 µl PBS in a microcentrifuge tube, followed by the addition of 10 µl cell suspension. After mixing all the constituents, 10 µl of the cell suspension was loaded onto the edge of the coverslip, which was consequently drawn into the chamber by capillary action. The haemocytometer was then immediately placed under a microscope to count the number of viable cells. The haemocytometer has two counting chambers consisting of nine 1 x 1 mm (1 mm²) squares each. The cells solely in the middle and corner squares were counted, including the cells that are touching or on the right and bottom lines of these squares. This means that cells in a total of 5 squares per side of the counting chamber were counted. The average number of viable cells per square was calculated. The average per square was then multiplied by the dilution factor (5×10^4) to determine the number of cells per ml of the cell suspension (C_1). Equation 3.1 was adapted and utilised to determine the dilution of the cell suspension required for seeding.

$$C_1V_1 = C_2V_2 \qquad (3.1)$$

Where:

C_1 = calculated cell concentration (cells/ml);

C_2 = cell density required for seeding (cells/ml);

V_2 = final volume of cell suspension required;

V_1 = volume of cell suspension to be added.

An automated cell counter (Scepter™) was used to determine the cell concentration of the 84BR cell suspension. Firstly, a dilution of the cell suspension was made by adding 900 µl of PBS to a 1.5 ml microcentrifuge tube followed by the addition of 100 µl cell suspension. Prior to cell counting, a sensor was attached to the end of the Scepter cell counter. Once the sensor was attached, the plunger was held down and the sensor was submerged in the prepared dilution. The plunger was released gradually while keeping the sensor submerged until the whole sample was loaded. The cell counting was done in triplicate and an average cell suspension concentration was calculated. Equation 3.1 was utilised to determine the dilution required to obtain the correct suspension density for seeding of the 84BR cells.

3.4.2 Methylthiazol tetrazolium (MTT) cytotoxicity assay

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water-soluble tetrazolium salt that is reduced in the mitochondria of metabolically active cells by succinate dehydrogenase enzymes to purple formazan crystals. The formation of these crystals is directly related to mitochondrial activity, which also indicates cell viability (Clothier *et al*., 2013:1350). The MTT assay determines cell viability by measuring the ability of healthy cells to convert yellow tetrazolium salt to purple formazan crystals, which can be dissolved with DMSO and quantified by spectrophotometry (Perez *et al*., 2017:43).

The HaCaT and 84BR cells were inoculated in 96-well plates at a seeding density of 100 000 cells/ml (20 000 cells/well) and 50 000 cells/ml (10 000 cells/well), respectively. Prior to treatment, the cells were incubated for 24 h in a humidified environment with 5% CO_2 at 37°C to form a monolayer of cells on the bottom of the well. After the 24 h culture period, the cells were treated daily with *A. vera* gel, *C. longa* extract (curcumin), *B. frutescens* aqueous extract or *B. frutescens* gel solutions respectively in the concentration ranges previously described. The MTT assay was performed after a 24 h and 48 h exposure time with each test solution. The MTT assay method used in this study was adapted from a method previously published by Fox *et al.* (2017:2) and Mazumder *et al.* (2016:285). The MTT solution (5 mg/ml) was freshly prepared in PBS on the day of the assay. At the beginning of the MTT assay, the test solutions were removed from the cells in each well and the cells were washed twice with 100 µl PBS. The dead cell control group was treated with 200 µl Triton-X (0.2% in PBS) for 15 min. A volume of 20 µl of the MTT solution and 180 µl non-supplemented DMEM were added to each well and the plates were

incubated for a period of 4 h in the dark. After incubation, the MTT solution was discarded and 200 µl of DMSO added to each well to dissolve the formazan crystals. The plates were incubated for a period of 1 h on a plate shaker and the absorbance was measured at wavelengths of 560 nm (signal) and 630 nm (background) with a SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA). DMSO was used as a blank.

The percentage cell viability was calculated by using Equation 3.2:

$$\%\ \text{cell viability} = \frac{\Delta\ \text{Absorbance of treated cells} - \Delta\ \text{Absorbance of blank}}{\Delta\ \text{Absorbance of untreated cells} - \Delta\ \text{Absorbance of blank}} \times 100 \qquad (3.2)$$

where Δ absorbance represents the 560 nm signal − 630 nm background

3.4.3 Neutral red (NR) cytotoxicity assay

The neutral red (NR) assay measures cell viability with regards to the ability of viable cells to absorb the NR dye, which accumulates in the lysosomes of living cells (Fotakis & Timbrell, 2006:172). The NR dye is transported into the lysosomes of the living cells by virtue of passive non-ionic diffusion (Perez *et al*., 2017:43). In the lysosomes, the NR dye attaches to the phosphate and/or anionic groups of the lysosomal matrix via electrostatic hydrophobic bonds. NR uptake is determined by the potential of the cell to preserve pH gradients by means of ATP production. The dye is permeable through cell membranes at a physiological pH due to its zero-net charge. The proton gradient inside the lysosomes creates a lower pH and consequently the dye becomes charged and becomes consequently unable to permeate out of the cell across the cell membrane (Repetto *et al*., 2008:1125).

The NR solution was prepared a day in advance of the assay, covered with aluminium foil and stored overnight in the incubator to equilibrate. NR has a tendency to precipitate when stored and needs to be filtered as the dye crystals may interfere with the assay (Sigma-Aldrich, 2018:2). The NR solution was therefore filtered before the assay through a 0.45 µm syringe filter to remove the dye crystals. This was followed by the addition of 200 µl NR solution to each well. According to the supplier guidelines (Sigma-Aldrich, 2018:1), NR stock solution (0.33%) should be added in an amount equivalent to 10% of the growth medium volume needed. The amount of NR stock solution needed was calculated by means of Equation 3.3.

Amount NR stock = Total volume of NR solution needed x 10% (3.3)

Next, the amount of non-additive DMEM that should be added to the NR solution was calculated utilising Equation 3.4.

Amount non-additive media = Total volume of NR solution – Amount NR stock (3.4)

The cell seeding densities and treatments for the NR assay were exactly the same as described for the MTT assay in section 3.4.2. The NR assay method used in this study was modified from a method previously published by Ouedraogo *et al.* (2014:3). At the start of the NR assay, the test solutions were removed from the cells in each well and discarded. The cells were washed twice with 100 µl PBS and 200 µl NR solution (0.033% in non-additive DMEM) were added to each well. The plate was then incubated for 2 h at 37°C, 5% CO_2 and 95% humidified air. After incubation, the NR solution was removed and 100 µl fixative solution (1% $CaCl_2$ in 0.5% formaldehyde) was added. Thereafter, the fixative solution was removed and 150 µl solubilisation solution (1% acetic acid in 50% ethanol) was added and the plate was incubated for 10 min on a shaker to dissolve the NR pink crystals. The absorbance was recorded at 540 nm (signal) and 690 nm (background) wavelengths making use of a SpectraMax® Paradigm® Multi-Mode Microplate reader (Molecular Devices, California, USA). Solubilisation solution was used as blank. The percentage cell viability was calculated by means of Equation 3.2, with the abovementioned wavelengths instead.

3.4.4 Wound healing scratch assay

The wound healing scratch assay method described for this study was adapted from methods previously published by Anitha *et al.* (2018:169) and Talekar *et al.* (2017:75). A cell suspension was prepared by trypsinisation of the HaCaT cells, followed by counting the cells with a haemocytometer as described in section 3.4.1. The cell suspension was diluted to 4 x 10^5 cells/ml with growth medium and added in 2.5 ml aliquots to each well of a 12-well plate. This was followed by incubation for a period of 24 h at 37˚C to obtain a monolayer of HaCaT cells. A sterile 200 µl micropipette-tip was then used to create a linear scratch (or wound gap) across the monolayer of the cells on the bottom of each well. This mechanical disruption of the monolayer of cells by means of a scratch imitates a wound in the skin. The wells were carefully rinsed with serum-free media four times to ensure that all dead cells and debris were removed from the well plates. Fresh growth medium containing the various test solutions (individual or combination solutions) were added to the wells in triplicate. The cells were incubated and photomicrographs of every well were taken at 24 h intervals for a total period of 48 h, to determine the wound healing potential of each individual test material. For the combinations thereof, photomicrographs of every well were taken at 12 h intervals for a total period of 48 h. The images were taken with a light microscope (Axiovert 25, Carl Zeiss Microscopy; New York, USA). Image J software was utilised to measure the wound surface area and the closure % and the migration rate were calculated by means of Equation 3.5 and 3.6, respectively:

$$\% \text{ wound closure} = \left[\frac{(\text{Pre} - \text{migration})_{\text{surface area}} - (\text{Migration})_{\text{surface area}}}{(\text{Pre} - \text{migration})_{\text{surface area}}}\right] \times 100 \quad (3.5)$$

$$\text{Migration rate } (\mu\text{m}^2/\text{h}) = \left[\frac{(\text{Pre} - \text{migration})_{\text{surface area}} - (\text{Migration})_{\text{surface area}}}{\text{Time (h)}}\right] \quad (3.6)$$

where (Pre-migration) is the initial wound surface area (μm^2) at time 0 h, while (Migration) is the wound surface area at a specific time (h).

3.5 Statistical data analysis

The data was statistically analysed with STATISTICA Version 13 (Statsoft, Tulsa, OK, USA). The wound healing and migration data sets were subjected to the Levene test of homogeneity of variances, to establish the normality distribution of the data. For normally distributed data, an analysis of variance (ANOVA) followed by the Tukey's Honest Significant Difference (HSD) post-hoc test were performed to identify significant differences ($p < 0.05$) between the treatments and the untreated control group. The non-parametric Kruskal-Wallis test was used as verification.

3.6 References

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CHAPTER 4 RESULTS AND DISCUSSION

4.1 Characterisation of plant materials

4.1.1 Chemical characterisation of *Aloe vera* gel with nuclear magnetic resonance spectroscopy

The ^{1}H-NMR spectrum acquired for *Aloe vera* gel (Daltonmax 700®) with the peaks allocated to selected marker molecules, is shown in Figure 4.1. The quantity of each marker molecule present in the *A. vera* gel (Daltonmax 700®), as determined by quantitative ^{1}H-NMR spectrometry is shown in Table 4.1.

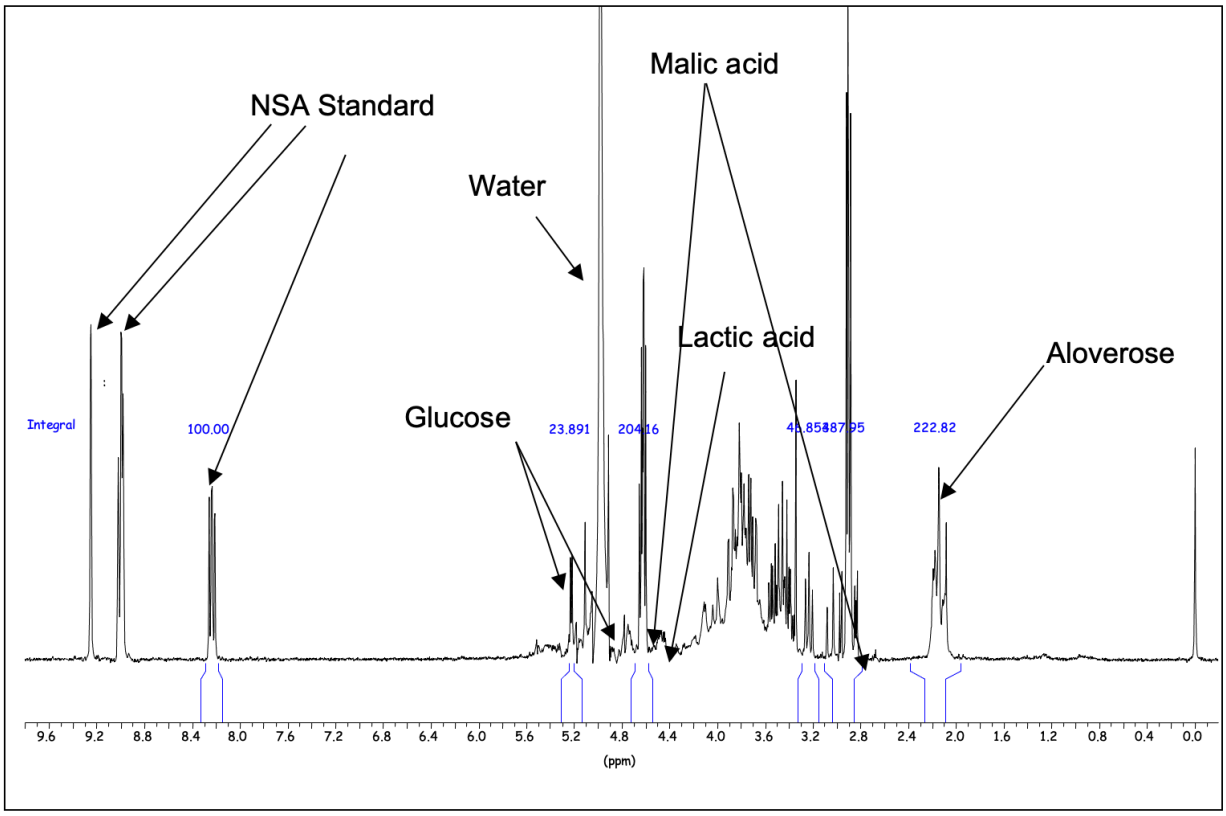


Figure 4.1: Proton nuclear magnetic resonance (^{1}H-NMR) spectrum of *A. vera* gel with peaks allocated to marker molecules

Table 4.1: Quantity of marker molecules present in the *A. vera* gel obtained from quantitative ^{1}H-NMR spectrometry

Component	Content (%)	Content (mg/l)
Aloverose (polysaccharide)	15.2	1065.8
Glucose	9.8	682.6
Malic acid	20.7	1446.1
Citric acid	2.0	140.0

The three main marker molecules that are used to confirm the identity of plant material to be of *A. vera* origin, namely aloverose, glucose and malic acid (Jiao *et al.,* 2010:842) were detected in the *A. vera* gel material used in this study. As indicated by the quantitative ^{1}H-NMR spectrometry, a low level of citric acid, which is a whole leaf marker molecule or added acidifier, was also present in the *A. vera* gel material (Daltonmax 700®). A total of 15.2 % aloverose, based on the weight of the material, was found to be present in the *A. vera* gel material (Daltonmax 700®) used in this study. Aloverose is a polysaccharide (i.e. partly acetylated polymannose) that has been identified as one of the biologically active molecules in *A. vera* gel (Jiao *et al.,* 2010:842; Beneke *et al.,* 2012:476).

4.1.2 Chemical fingerprinting of *Bulbine frutescens* gel and aqueous extract with nuclear magnetic resonance spectroscopy

The nuclear magnetic resonance (both ^{1}H-NMR and ^{13}C-NMR) spectra for *B. frutescens* gel and aqueous extract are shown in Figures 4.2 and 4.3, respectively. A qualitative analytical method was utilised to obtain the chemical fingerprints of these plant materials to indicate differences in their chemical composition.

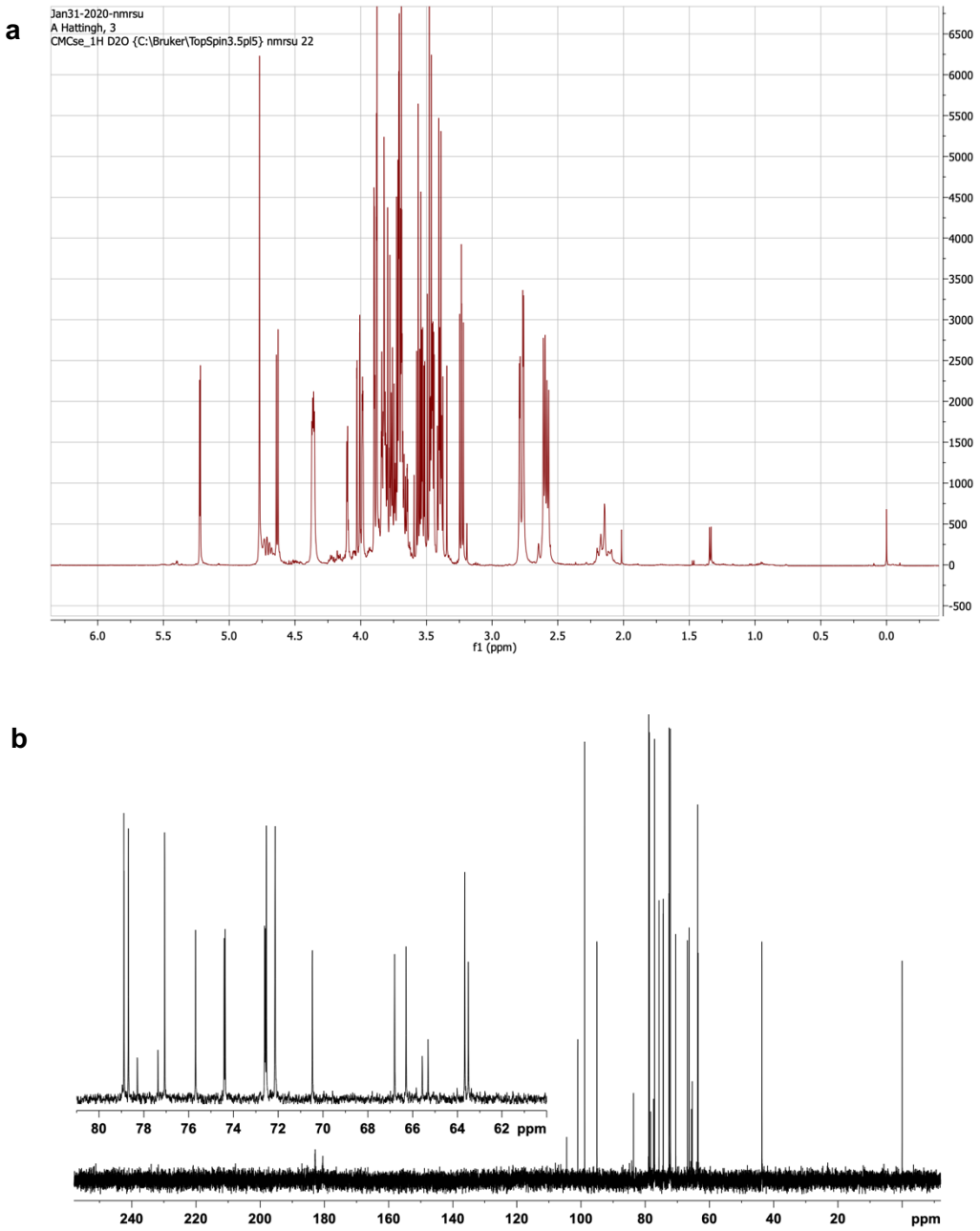


Figure 4.2: The ^{1}H-NMR (a) and ^{13}C-NMR (b) spectra for *B. frutescens* gel.

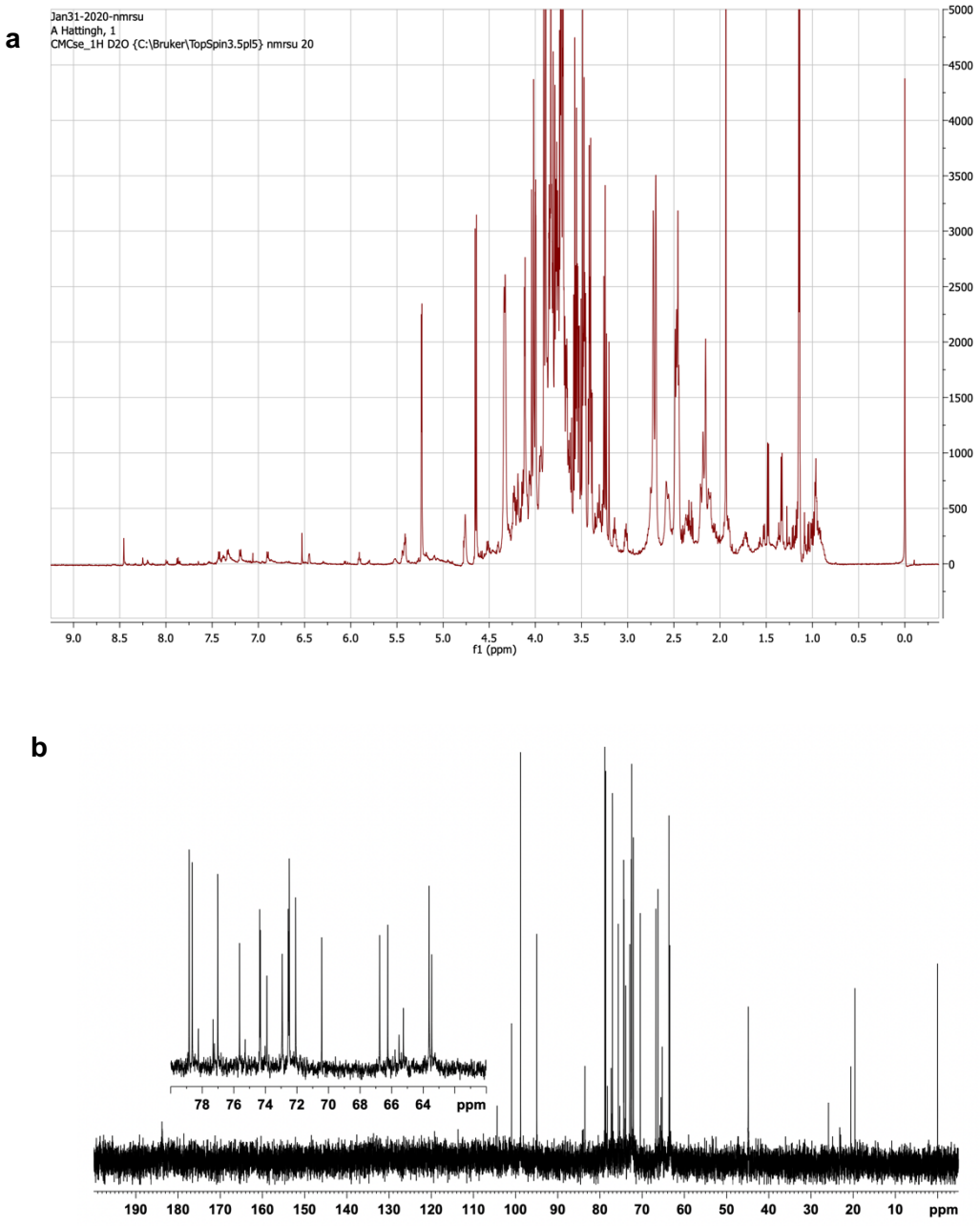


Figure 4.3: The ^{1}H-NMR (a) and ^{13}C-NMR (b) spectra for *B. frutescens* aqueous extract.

It is evident from the ^{1}H-NMR and ^{13}C-NMR spectra in Figures 4.2 and 4.3 that the phytochemical composition of *B. frutescens* gel and aqueous extract have some phytochemical overlap (as indicated by the same peaks in each of the material's NMR spectra), but different chemical components are also present in each extracted material (as indicated by different peaks in each material's NMR spectra). Further chemical analysis of the phytochemical composition of *B.*

frutescens aqueous extract and gel is necessary in order to be specific with respect to the chemical differences between these two materials.

4.1.3 Chemical characterisation of *Curcuma longa* extract (curcumin)

4.1.3.1 Analytical method verification: linearity

In order to verify the HPLC analytical method used to determine the curcumin raw material's purity, the linearity of the correlation between the peak area of the chromatograms and the curcumin concentration was determined. The graph of the area under the peak plotted as a function of the concentration range of curcumin is shown in Figure 4.4.

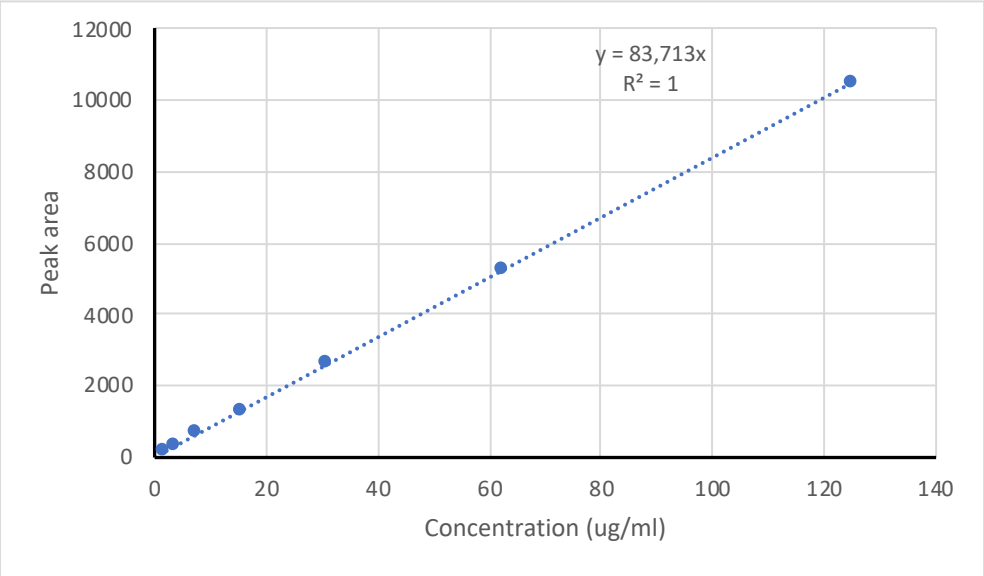


Figure 4.4: Graph of peak area plotted as a function of curcumin concentration unto which linear regression was applied

A correlation coefficient (R^2) value of 1 was obtained for the *C. longa* extract (curcumin) graph where peak area was plotted as a function of curcumin concentration, which is an indication that the method for curcumin quantification complies with the linearity specification. According to Singh (2013:29), an R^2 value higher than 0.998 indicates an acceptable correlation between peak area and analyte concentration.

4.1.3.2 Curcumin raw material purity

The purity of the raw material of *C. longa* extract (or curcumin) used in this study, in comparison to the curcumin reference standard, is shown in Table 4.2.

Table 4.2: The percentage purity of the raw material of *C. longa* extract (curcumin) analysed against a curcumin reference standard

Purity (%)		
Sample 1	Sample 2	Sample 3
101.77%	102.56%	102.84%

The results in Table 4.2 show that the curcumin used in this study is chemically pure and complied with the in-house purity limit of 100 ± 5%, in comparison to the reference standard.

4.2 Cytotoxicity asssays

López-García *et al.* (2014:44) suggested a classification system for *in vitro* cytotoxicity assay results to indicate the level of cytotoxicity based on the percentage cell viability as shown in Table 4.3.

Table 4.3: Classification of cytotoxicity in relation to percentage cell viability

Cytotoxicity description	Strong	Moderate	Weak	Non-cytotoxic
Cell viability (%)	<40	40-60	60-80	>80

4.2.1 Methyl thiazolyl tetrazolium (MTT) assay

The MTT cytotoxicity assay results for *A. vera* gel are shown in Figure 4.5, while the MTT cytotoxicity assay results for *C. longa* extract (curcumin) are displayed in Figure 4.6 and those for *B. frutescens* aqueous extract and *B. frutescens* gel are shown in Figures 4.7 and 4.8, respectively.

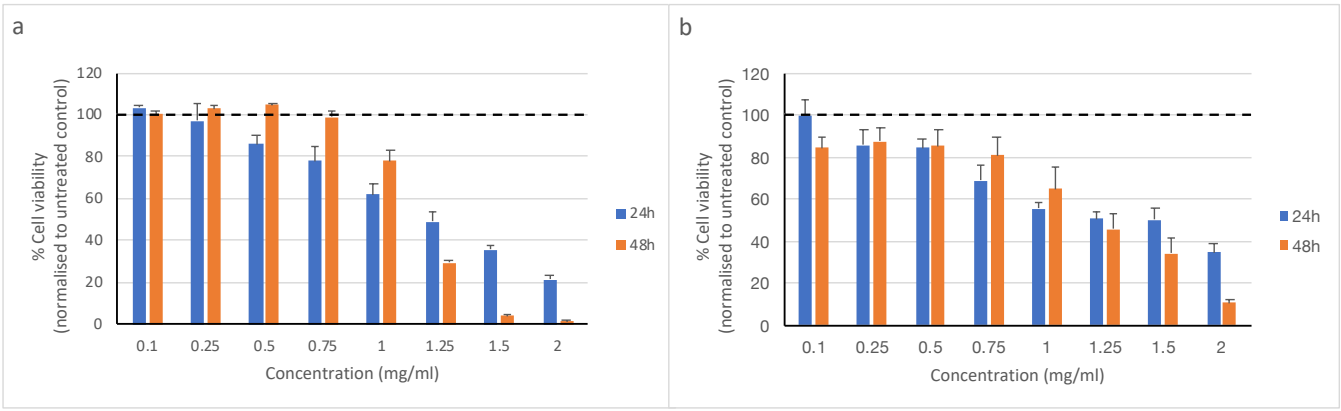


Figure 4.5: Percentage viability of HaCaT (a) and 84BR (b) cells treated with *A. vera* gel for periods of 24 h and 48 h over a concentration range of 0.1 to 2 mg/ml, as determined with the MTT assay. The columns represent the mean viability values (n = 6) of the cells and the error bars indicate the standard deviations. All the data were normalised to the untreated control, which is considered as 100% viable (indicated on the graph by the dashed line).

Figure 4.5 shows that a concentration dependent decrease in cell viability for both HaCaT and 84BR cells occurred with exposure to *A. vera* gel over a period of 24 h and 48 h. The *A. vera* gel started to exhibit weakly toxic properties above a concentration of 0.5 mg/ml. These findings are in conformity with previous studies on the cytotoxicity of *A. vera* gel (Du Plessis and Hamman, 2014:172; Fox *et al.,* 2017:4).

Interestingly, the *A. vera* gel applied at concentrations of 0.25, 0.5, 0.75 and 1 mg/ml showed a lower effect on cell viability at an exposure time of 48 h for both HaCaT and 84BR cell lines than to an exposure time of 24 h. From the MTT assay results at 48 h, it is clear that cell viability dropped below 80% only at concentrations above 0.75 mg/ml (López-García *et al*., 2014:44). Based on these results, concentrations of *A. vera* gel between 0.25 and 0.75 mg/ml were considered to be safe for use in the wound healing studies.

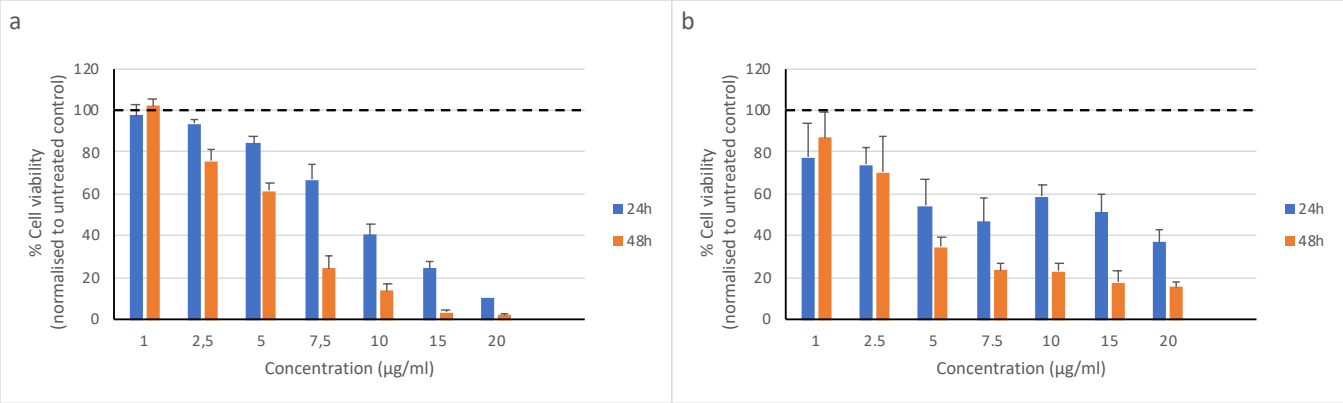


Figure 4.6: Percentage cell viability of HaCaT (a) and 84BR (b) cells treated with *C. longa* extract (curcumin) for periods of 24 h and 48 h over a concentration range of 1 to 20 µg/ml, as determined with the MTT assay. The columns represent the mean viability values (n = 6) of the cells and the error bars indicate the standard deviations. All the data were normalised to the untreated control, which is considered as 100% viable (indicated on the graph by the dashed line).

It is clear from the MTT assay results presented in Figure 4.6 that moderate to strong cell viability reduction occurred after exposure to *C. longa* extract (curcumin). The percentage cell viability of both the HaCaT and 84BR cells decreased with an increase in the curcumin concentration. At higher concentrations (> 7.5 µg/ml), curcumin demonstrated strong cytotoxicity in both the HaCaT and 84BR cell models (López-García *et al*., 2014:44). These results are in accordance with the findings from previous studies where the cytotoxicity results of curcumin showed a concentration dependent decrease in cell viability (Sharma *et al.,* 2018:1012; Zhao *et al.,* 2013:3). Curcumin previously also exhibited apoptosis in fibroblasts at a relatively high treatment concentration (25 µm) (Scharstuhl *et al.,* 2009:715).

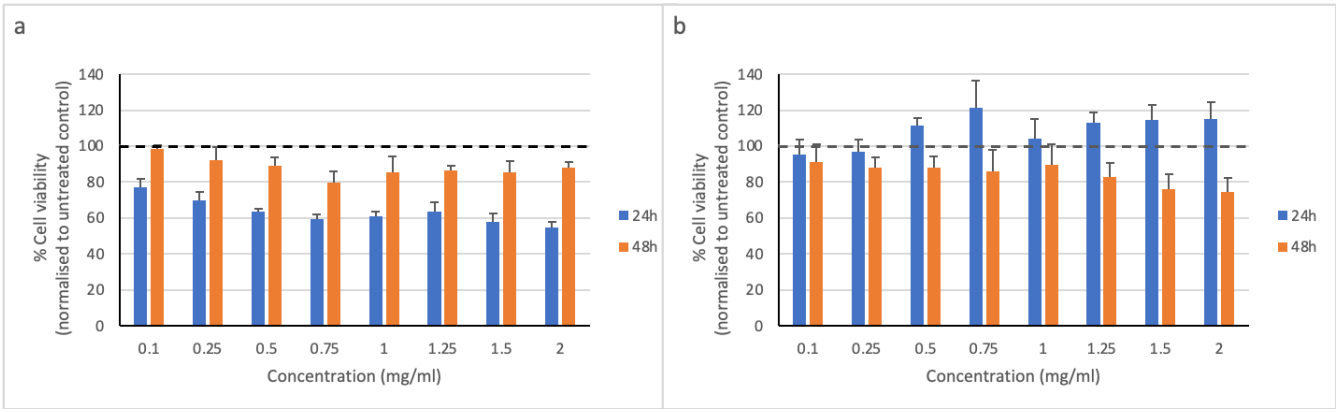


Figure 4.7: Percentage cell viability of HaCaT (a) and 84BR (b) cells treated with *B. frutescens* aqueous extract for periods of 24 h and 48 h over a concentration range of 0.1 to 2 mg/ml, as determined with the MTT assay. The columns represent the mean viability values (n = 6) of the cells and the error bars indicate the standard deviations. All the data were normalised to the untreated control, which is considered as 100% viable (indicated on the graph by the dashed line).

B. frutescens extract showed a lower decrease in cell viability after a 48 h exposure period to the HaCaT cell line as compared to the cell viability obtained after a 24 h exposure period. In fact, no cytotoxicity (i.e. a percentage cell viability > 80%) was observed at all the concentrations of *B. frutescens* extract treatment after a 48 h exposure period. On the other hand, an opposite effect was observed in the 84BR cell line, where the cell viability was not affected after 24 h of exposure time, while a relatively higher decrease in cell viability occurred after the 48 h exposure period. From the MTT assay results, it is clear that no to weak cytotoxicity (i.e. cell viability > 60%) was obtained for *B. frutescens* aqueous extract for all the concentrations applied in this study (López-García *et al*., 2014:44). Therefore, the selected treatment concentrations of 0.25, 0.5 and 0.75 mg/ml for *B. frutescens* aqueous extract were considered to be safe for use in the wound healing studies.

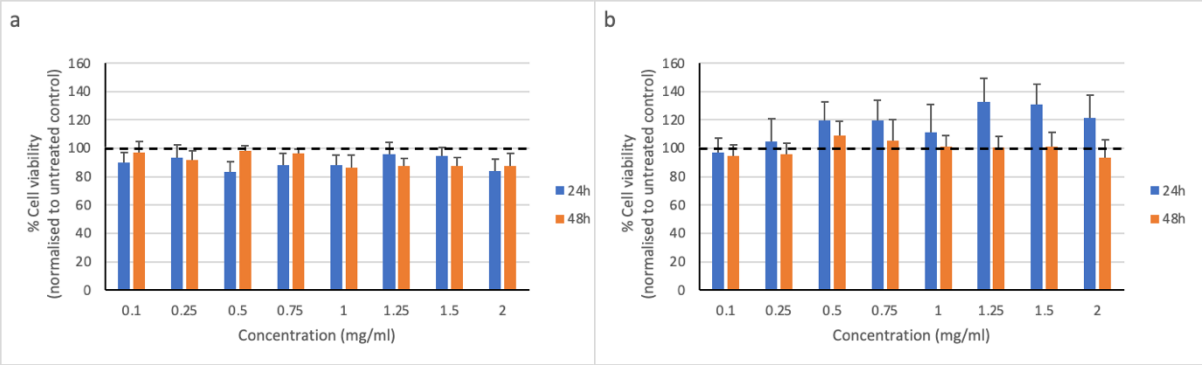


Figure 4.8: Percentage cell viability of HaCaT (a) and 84BR (b) cells treated with *B. frutescens* gel for periods of 24 h and 48 h over a concentration range of 0.1 to 2 mg/ml, as determined with the MTT assay. The columns represent the mean viability values (n = 6) of the cells and the error bars indicate the standard deviations. All the data were normalised to the untreated control, which is considered as 100% viable (indicated on the graph by the dashed line).

From the MTT results, it is apparent that all the concentrations of *B. frutescens* gel applied demonstrated a percentage cell viability above 80% after 24 h and 48 h, which can be classified as non-cytotoxic according to López-García *et al.* (2014:44). Therefore, the selected concentrations of *B. frutescens* gel (0.25, 0.5 and 0.75 mg/ml) for use in the wound healing studies were considered to be safe.

As previously mentioned, interference with the MTT assay can occur when investigating plant extracts that contain multiple phytochemical compounds. In a study by Shikalepo *et al*. (2018:76), a phytochemical screening of *B. frutescens* extract was performed and phytochemicals with good antioxidant activity such as phenols, alkaloids and flavonoids were identified. These phytochemicals can potentially cause interference with the MTT assay resulting in false positive cytotoxic test results. Phytochemicals such as antioxidants and polyphenols have the capability of reducing yellow tetrazolium salt to formazan crystals that can lead to unreliable results (Van Tonder *et al.,* 2015:2). According to Maioli *et al*. (2009:238), polyphenols possess mitochondrial uncoupling properties that can lead to overvaluation of MTT cell viability results. Therefore, neutral red (NR) toxicity cell assays were also performed in this study as an alternative assay to evaluate the cytotoxicity of the selected plant materials. By performing more than one cytotoxicity assay, the reliability of the cell viability results is increased (Fotakis & Timbrell, 2006:177).

4.2.2 Neutral red (NR) assay

The NR assay results for *A. vera* gel, *C. longa* extract (curcumin), *B. frutescens* aqueous extract and *B. frutescens* gel are shown in Figures 4.9 to 4.12, respectively.

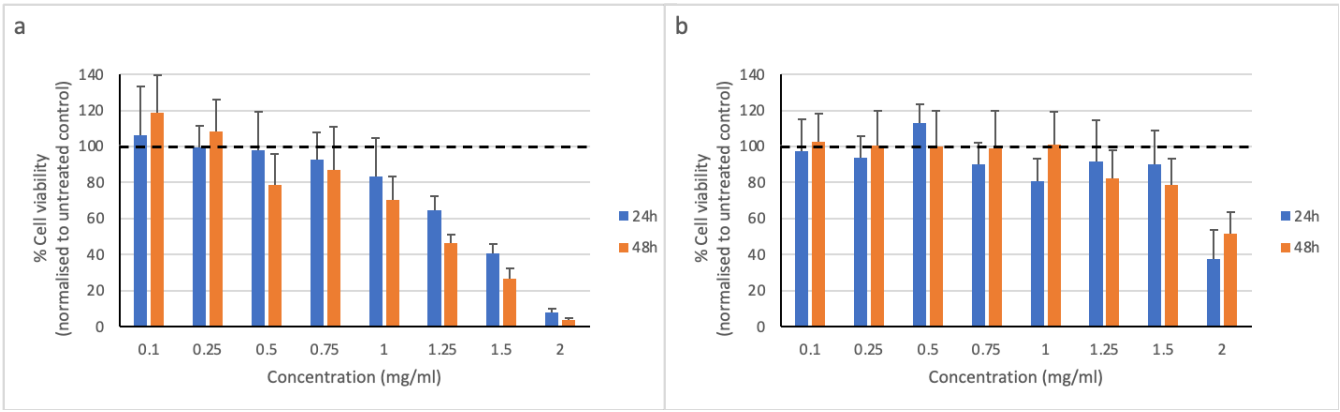


Figure 4.9: Percentage cell viability of HaCaT (a) and 84BR (b) cells treated with *A. vera* gel for periods of 24 h and 48 h over a concentration range of 0.1 to 2 mg/ml, as determined with the NR assay. The columns represent the mean viability values (n = 6) of the cells and the error bars indicate the standard deviations. All the data were normalised to the untreated control, which is considered as 100% viable (indicated on the graph by the dashed line).

The HaCaT cell line treated with *A. vera* gel showed a concentration dependent decrease in cell viability. The same concentration dependent decrease in cell viability could, however, not be observed within the 84BR cell line. Especially at higher concentrations (> 1 mg/ml), the *A. vera* gel had a less toxic effect on the 84BR cell line than on the HaCaT cell line. From the NR results (Figure 4.9), it is clear that no to weak cytotoxicity (i.e. cell viability > 60%) can be observed at *A. vera* gel concentrations < 1 mg/ml (López-García *et al*., 2014:44). Therefore, the NR cytotoxicity results support the MTT cytotoxicity results, which both indicated that *A. vera* gel concentrations of 0.25, 0.5 and 0.75 mg/ml are considered safe to use in the wound healing studies.

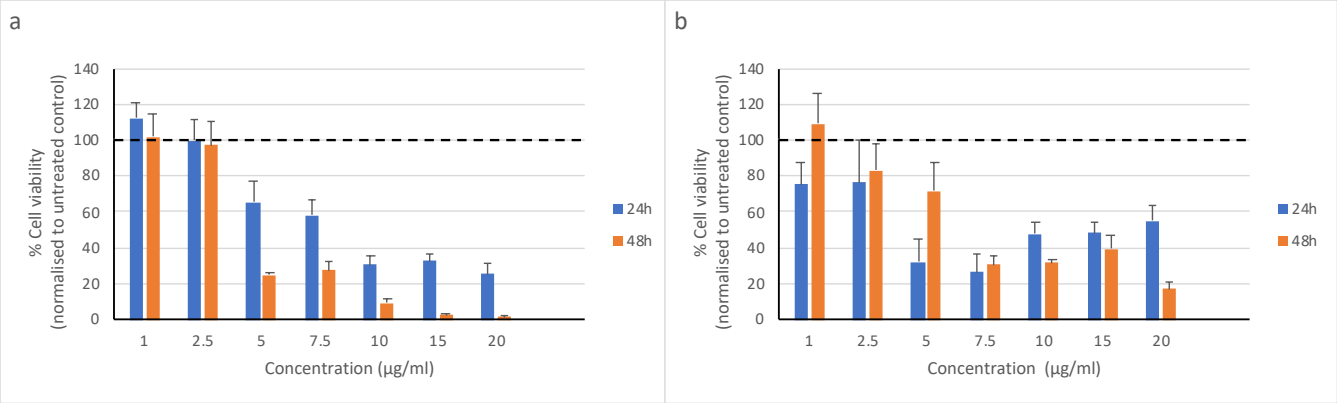


Figure 4.10: Percentage cell viability of HaCaT (a) and 84BR (b) cells treated with *C. longa* extract (curcumin) for periods of 24 h and 48 h over a concentration range of 1 to 20 µg/ml, as determined with the NR assay. The columns represent the mean viability values (n = 6) of the cells and the error bars indicate the standard deviations. All the data were normalised to the untreated control, which is considered as 100% viable (indicated on the graph by the dashed line).

According to the NR cytotoxicity assay results, a concentration dependent decrease in cell viability for both HaCaT and 84BR cell lines was observed when treated with *C. longa* extract (curcumin). At the higher treatment concentrations applied in this study (> 2.5 µg/ml), strong cytotoxicity effects were obtained (López-García *et al*., 2014:44). Therefore, only the three lowest concentrations (1; 2.5 and 5 µg/ml) of curcumin that demonstrated the highest cell viability, were utilised for the wound healing studies, even though cytotoxic effects were observed for these concentrations, especially at 48 h.

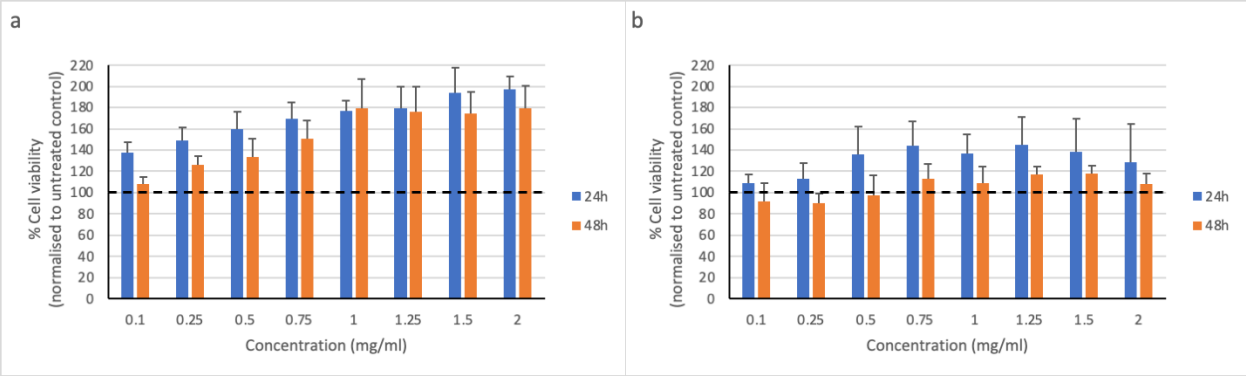


Figure 4.11: Percentage cell viability of HaCaT (a) and 84BR (b) cells treated with *B. frutescens* extract for periods of 24 h and 48 h over a concentration range of 0.1 to 2 mg/ml, as determined with the NR assay. The columns represent the mean viability values (n = 6) of the cells and the error bars indicate the standard deviations. All the data were normalised to the untreated control, which is considered as 100% viable (indicated on the graph by the dashed line).

Figure 4.11 illustrates the percentage cell viability of HaCaT and 84BR cells when treated with *B. frutescens* aqueous extract for 24 h and 48 h, as determined with the NR assay. Both cell lines showed higher cell viability values at higher treatment concentrations (i.e. the *B. frutescens* extract exhibited an apparent stimulation effect on cell division and growth). From the NR assay results, it is clear that no cytotoxicity (López-García *et al.*, 2014:44) was observed for all the concentrations of *B. frutescens* aqueous extract applied to the two selected cell lines in this study. Accordingly, concentrations of 0.25, 0.5 and 0.75 mg/ml were considered safe to be used in the wound healing studies, as also observed from the MTT findings. Furthermore, these results indicate that *B. frutescens* aqueous extract may potentially have very good wound healing properties, since it stimulates cell propagation and growth and does not cause any cytotoxic effects (Biranje *et al.,* 2020:8).

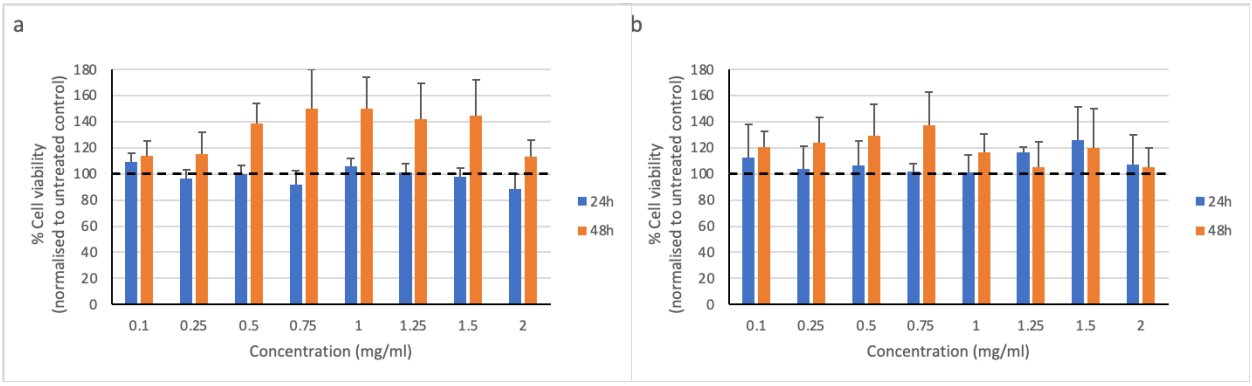


Figure 4.12: Percentage cell viability of HaCaT (a) and 84BR (b) cells treated with *B. frutescens* gel for periods of 24 h and 48 h over a concentration range of 0.1 to 2 mg/ml, as determined with the NR assay. The columns represent the mean viability values (n = 6) of the cells and the error bars indicate the standard deviations. All the data were normalised to the untreated control, which is considered as 100% viable (indicated on the graph by the dashed line).

From Figure 4.12, it is clear that no cytotoxicity (i.e. cell viability > 80%) was observed at all experimental concentrations of *B. frutescens* gel applied to the HaCaT and 84BR cells over a period of 24 h and 48 h. These findings are in line with the MTT cytotoxicity assay results obtained for *B. frutescens* gel, confirming its very low cytotoxic potential on the selected cell lines. The results also indicated a stimulation effect on skin cell propagation and growth, which may indicate high wound healing potential (Biranje *et al.,* 2020:8).

4.2.3 Vehicle control: 0.5% *v/v* dimethyl sulphoxide (DMSO)

The DMSO concentration in the *C. longa* extract (curcumin) test solutions, to which the HaCaT and 84BR cells were exposed, was kept below 0.5% *(v/v)*. A vehicle control group consisting of both HaCaT and 84BR cells treated with 0.5% *(v/v)* DMSO in growth medium for 24 h and 48 h was included in the MTT and NR cytotoxicity assays and the results are shown in Figure 4.13.

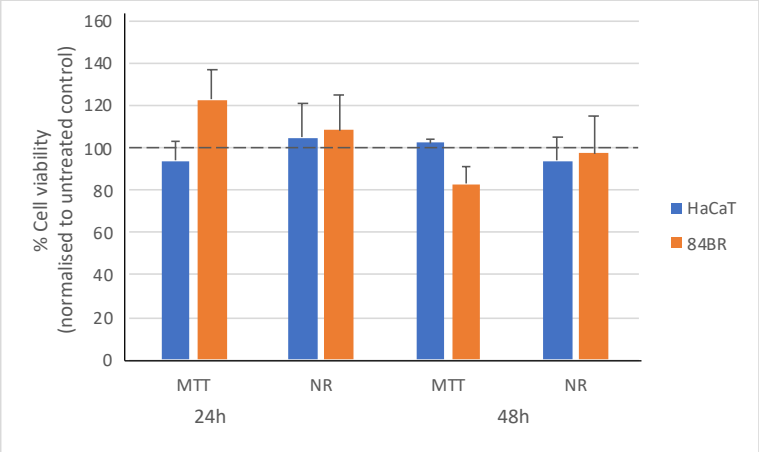


Figure 4.13: The percentage cell viability of the HaCaT and 84BR cell lines treated with 0.5% *(v/v)* DMSO in growth medium for 24 h and 48 h, as determined with the MTT and NR assays. The columns represent the mean viability values (n = 6) of the cells with error bars indicating the standard deviations. All the data are expressed as a percentage of the untreated control, considered as 100% viable, as indicated with the dashed line.

The vehicle control group demonstrated a cell viability of 80% or higher in both cell lines after 24 h and 48 h exposure periods, as determined with the MTT and NR cytotoxicity assays. According to López-García *et al.* (2014:44), the 0.5% *(v/v)* DMSO can therefore be considered as non-cytotoxic. From these results, it was clear that 0.5% *(v/v)* DMSO could be considered as safe and was therefore utilised to prepare the *C. longa* extract (curcumin) stock solutions as previously described in the "Methods" chapter.

4.3 Wound healing scratch assay

4.3.1 Individual plant materials

4.3.1.1 *A. vera* gel

Figure 4.14 shows the percentage wound closure and migration rate ($\mu m^2/h$) for *A. vera* gel at 24 h and 48 h exposure time periods towards HaCaT cells where the experimental values are expressed as a ratio to the untreated control group (therefore, the untreated control = 1). Microscopic images illustrating the wound areas induced by the scratch technique on HaCaT cells after 24 h and 48 h treatment with *A. vera* gel are depicted in Figure 4.15

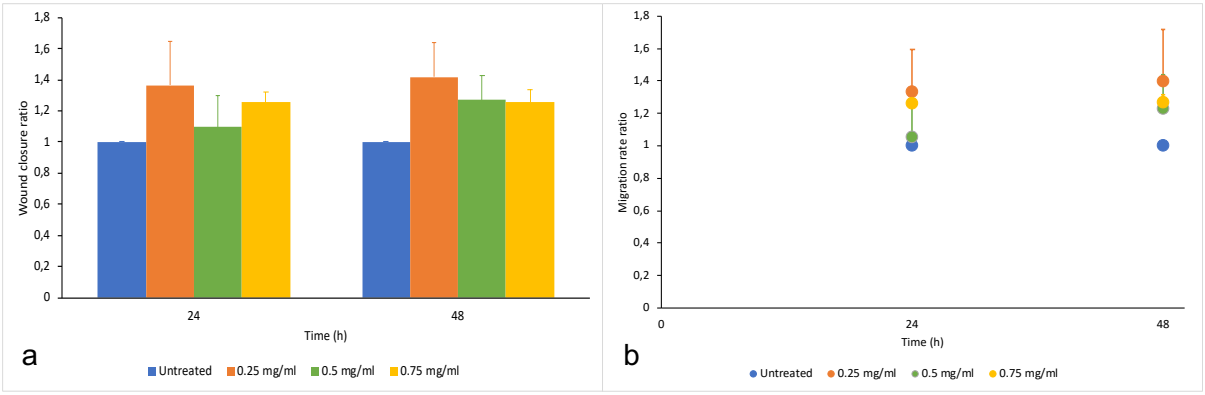


Figure 4.14: The percentage wound closure (a) and migration rate ($\mu m^2/h$) (b) results expressed as a ratio of the untreated control group of the HaCaT cells treated with *A. vera* gel at three concentrations over periods of 24 h and 48 h. All the data was normalised to the untreated control, which was considered as 1, as indicated on the graphs. The bars and markers represent the mean wound closure and migration rate ratios (n = 3) with error bars indicating the standard deviations. Any statistical significant differences with the untreated control are indicated on the graph as determined with Tukey's HSD (*p<0.05) and Kruskal-Wallis (**p<0.05) tests.

From the wound closure and migration rate ratio values obtained, it is clear that *A. vera* gel exhibited improved wound healing when compared to the untreated control group. The cells treated with all three *A. vera* gel concentrations showed a higher wound closure and cell migration rate ratio compared to that of the untreated control group, although it was not statistically significant. As the wound area became smaller after 24 h, the migration rate slowed down and reached a plateau between 24 h and 48 h. These findings are in line with previous findings where *A. vera* gel has been proven to enhance wound healing within *in vitro* cell culture models (Fox *et al.,* 2017:4-6).

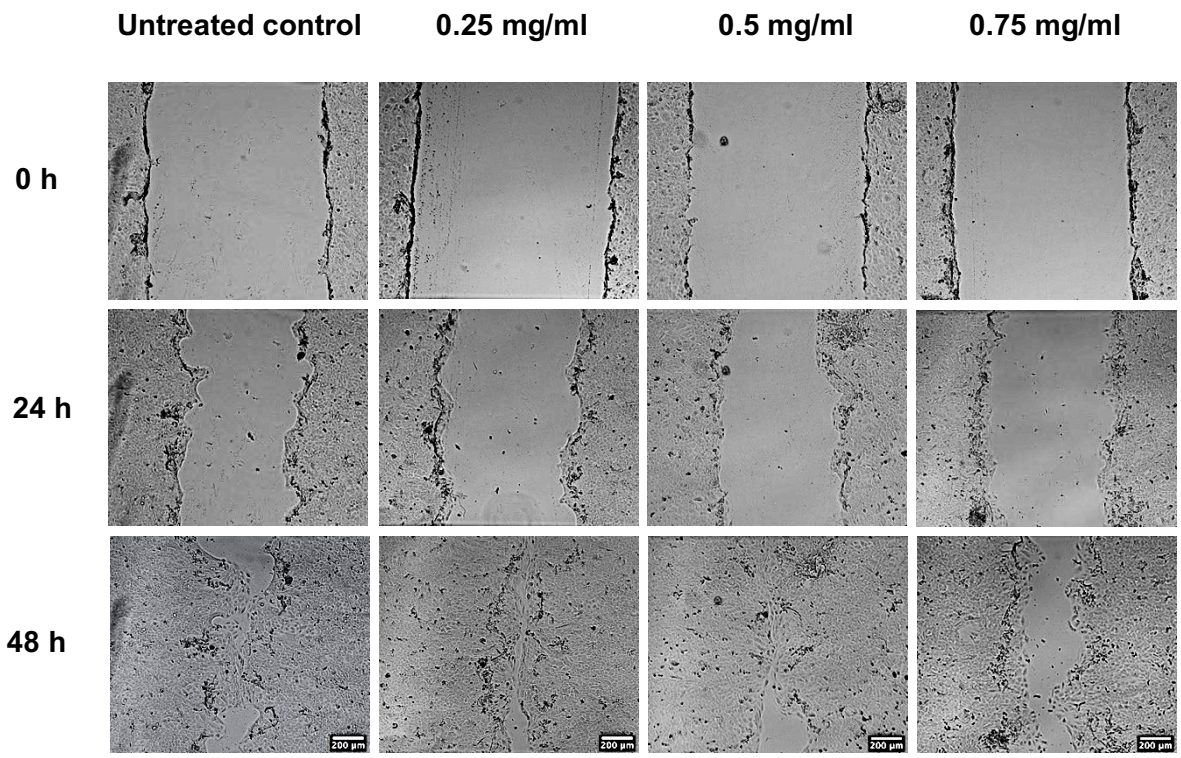


Figure 4.15: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 0.25 mg/ml, 0.5 mg/ml and 0.75 mg/ml *A. vera* gel for time periods of 0; 24 and 48 h. Images were captured at 10x magnification and scale bars show 200 µm.

4.3.1.2 *C. longa* extract (curcumin)

Figure 4.16 depicts the percentage wound closure and migration rate ($\mu m^2/h$) for curcumin at 24 h and 48 h exposure time periods towards HaCaT cells where the experimental values are expressed as a ratio to the untreated control group (therefore, the untreated control = 1). Microscopic images illustrating the wound areas induced by the scratch technique on HaCaT cells after 24 h and 48 h treatment with *C. longa* extract (curcumin) are depicted in Figure 4.17.

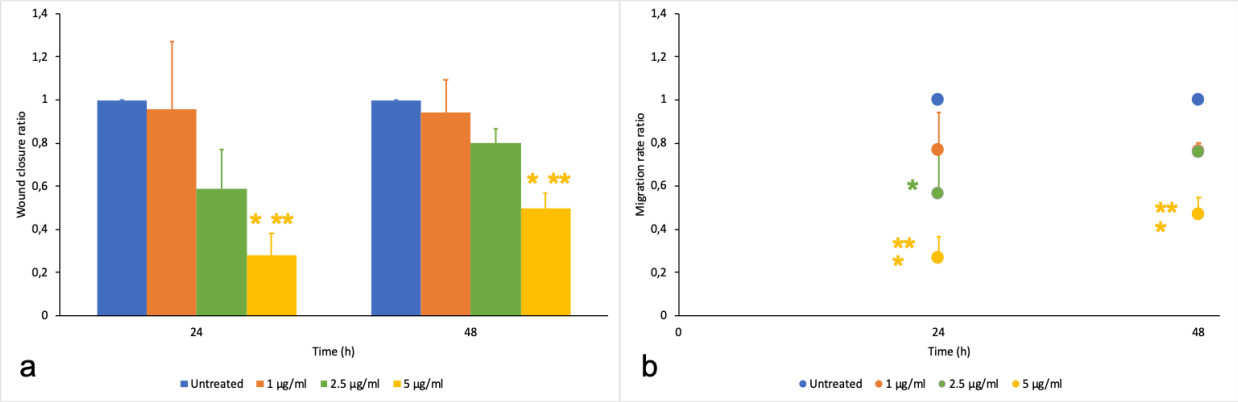


Figure 4.16: The percentage wound closure (a) and migration rate ($\mu m^2/h$) (b) results expressed as a ratio of the untreated control group of the HaCaT cells treated with *C. longa* extract (curcumin) at three concentrations over periods of 24 h and 48 h. All the data was normalized to the untreated control, which was considered as 1, as indicated on the graphs. The bars and markers represent the mean wound closure and migration rate ratios (n = 3) with error bars indicating the standard deviations. Any statistical significant differences with the untreated control are indicated on the graph as determined with Tukey's HSD (*p<0.05) and Kruskal-Wallis (**p<0.05) tests.

In contrast to the favourable wound healing results acquired with the other plant materials investigated in this study, none of the *C. longa* extract (curcumin) concentrations that were tested resulted in improved wound closure compared to that of the untreated control group (Figure 4.16). The HaCaT cells exposed to 5 µg/ml curcumin showed a significant decrease in percentage wound closure and migration rate ratio at 24 h and 48 h treatment periods when compared to the untreated control. At 24 h, 2.5 µg/ml also demonstrated a significant decrease in migration rate ratio. These findings correspond with the MTT and NR cytotoxicity results, as mentioned in Section 4.2, which demonstrated a concentration dependent decrease in cell viability after curcumin exposure.

This is in agreement with the results obtained in a previous study by Topman *et al.* (2013:170), where an *in vitro* wound healing model was used to investigate the effect of natural medications, such as curcumin, on the migration kinetics of fibroblasts. The findings showed that it had no impact on the kinematics of the fibroblasts. However, many *in vivo* studies have been conducted previously where curcumin has been found to promote wound healing through various mechanisms in the multi-faceted process of wound healing (Sidhu *et al*., 1998:176; Sidhu *et al.,*

1999:372; Panchatcharam *et al.,* 2006:9; Gadekar *et al.,* 2012:229; Ghosh & Gaba, 2013:767; Akbik *et al.,* 2014:2; Yen *et al.,* 2018:612). The negative results may be attributed to the difficulty to imitate the wound healing process *in vitro*, since it is a very complex process and cell migration depends on multiple factors (Topman *et al.,* 2013:172). Curcumin, as well as other natural products, can still influence the complex wound healing process *in vivo* through alternative pathways, because of its anti-inflammatory, antioxidant and antimicrobial properties (Maheshwari *et al*., 2006:2082; Topman *et al.,* 2013:170).

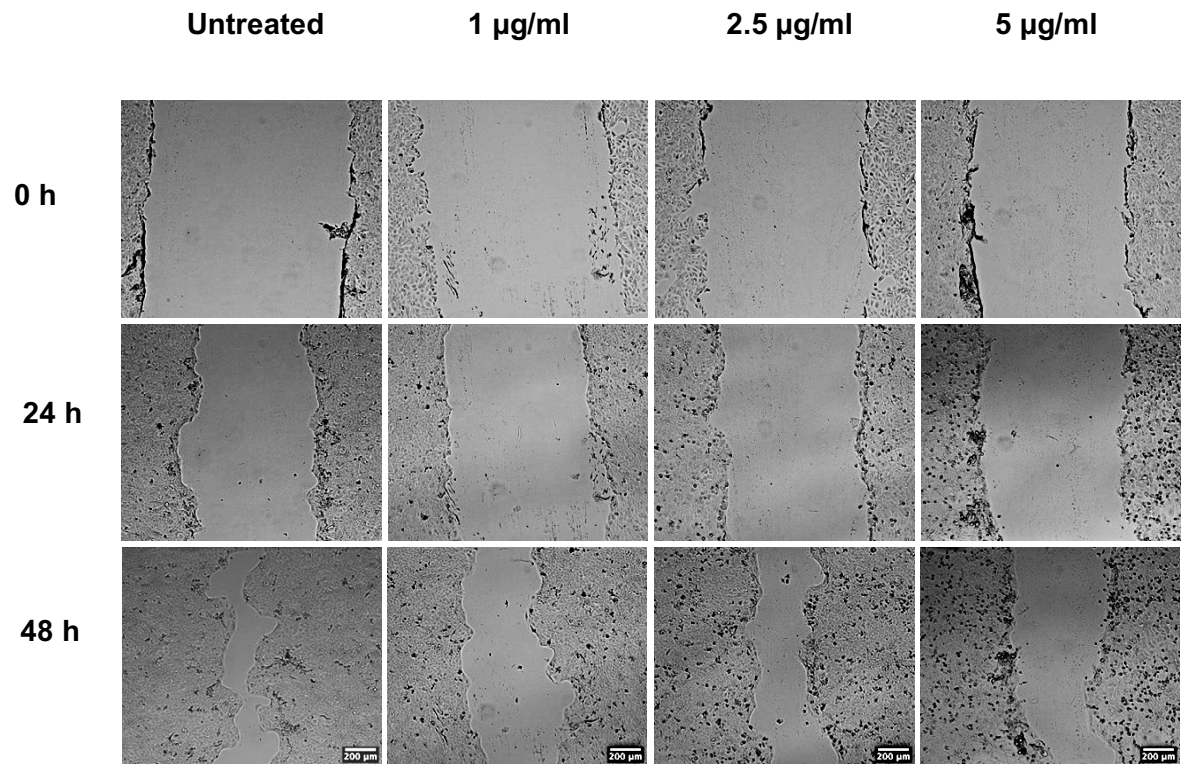


Figure 4.17: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 1 µg/ml, 2.5 µg/ml and 5 µg/ml *C. longa* extract (curcumin) for time periods of 0; 24 and 48 h. Images were captured at 10x magnification and scale bars show 200 µm.

4.3.1.3 *B. frutescens* aqueous extract

Figure 4.18 shows the percentage wound closure and migration rate ($\mu m^2/h$) for *B. frutescens* aqueous extract at 24 h and 48 h exposure time periods towards HaCaT cells where the experimental values are expressed as a ratio of the untreated control group (therefore, the untreated control = 1). Microscopic images illustrating the wound areas induced by the scratch

technique on HaCaT cells after 24 h and 48 h treatment with *B. frutescens* aqueous extract are depicted in Figure 4.19.

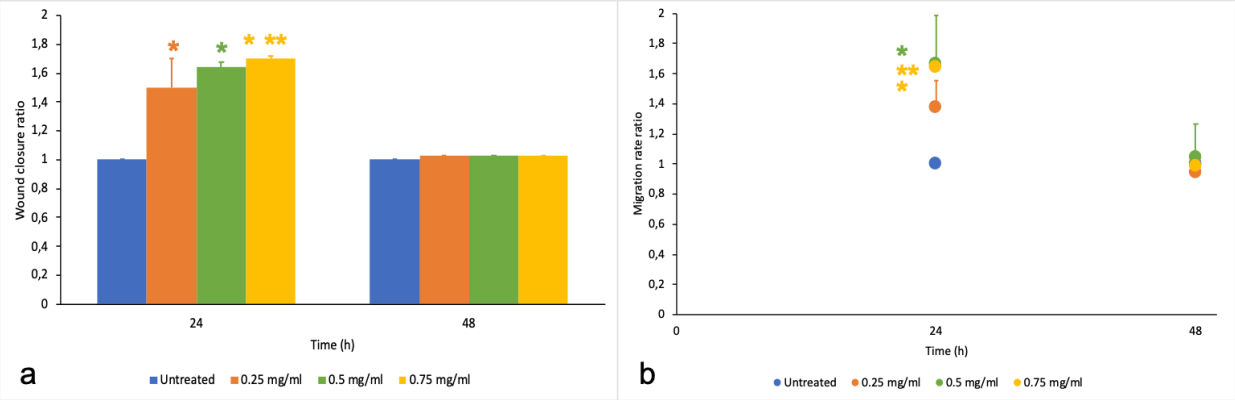


Figure 4.18: The percentage wound closure (a) and migration rate (μm^2/h) (b) results expressed as a ratio of the untreated control group of the HaCaT cells treated with *B. frutescens* aqueous extract at three concentrations over periods of 24 h and 48 h. All the data was normalised to the untreated control, which was considered as 1, as indicated on the graphs. The bars and markers represent the mean wound closure and migration rate ratios (n = 3) with error bars indicating the standard deviations. Any statistical significant differences with the untreated control are indicated on the graph as determined with Tukey's HSD (*p<0.05) and Kruskal-Wallis (**p<0.05) tests.

At 24 h, all three the *B. frutescens* aqueous extract concentrations showed significantly higher wound healing activity with ratios varying from 1.5 to 1.7, in comparison to the untreated control group. At 48 h, the wounds were almost completely closed in all the experimental groups, which was also evident in the untreated control group (Figure 4.18a). The *B. frutescens* extract treatment concentrations showed significant differences in wound closure ratios at 24 h when compared to the untreated control, exhibiting p-values of 0.000176 (0.25 mg/ml), 0.000175 (0.5 mg/ml) and 0.000175 (0.75 mg/ml), respectively (Tukey's HSD test). On the other hand, only the two highest treatment concentrations of *B. frutescens* extract showed a significant increase in the migration rate ratio at 24 h, relative to the untreated control (Figure 4.18b). The migration rate ratio decreased at 48 h, which can be explained by cell contact inhibition as a consequence of the almost complete wound closure percentages found at 48 h. As the cells proceed to move towards one another and the wound gap becomes smaller, the migration rate decelerates, due to an inhibitory force exerted by cell-to-cell contact (Puliafito *et al*., 2012:5-7).

From these wound healing results, it can be deduced that *B. frutescens* aqueous extract has the ability to increase the initial wound healing activity (over the first 24 h period), as compared to the untreated control group. One of the mechanisms to increase wound healing is by increasing the rate of cell migration as each phase of the wound healing process involves the migration of particular cells to interact with other cells in the wound environment (Topman *et al.,* 2013:172).

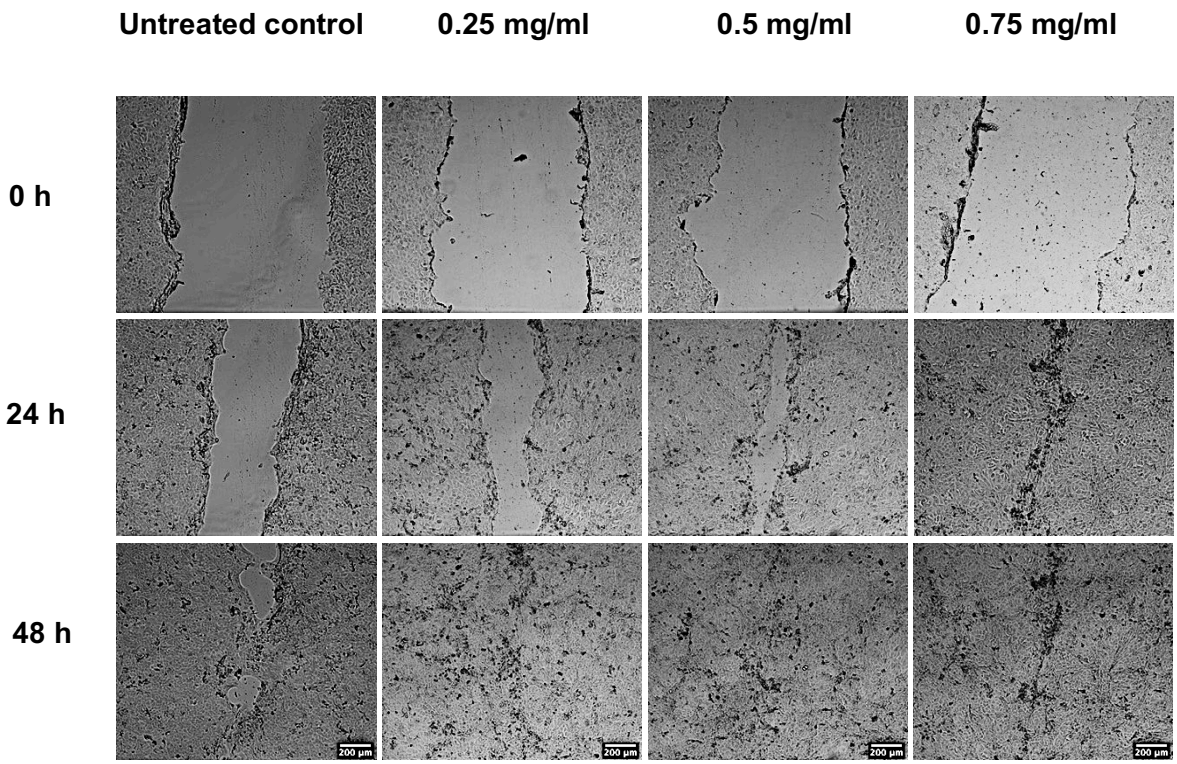


Figure 4.19: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 0.25 mg/ml, 0.5 mg/ml and 0.75 mg/ml *B. frutescens* extract for time periods of 0; 24 and 48 h. Images were captured at 10x magnification and scale bars show 200 µm.

4.3.1.4 *B. frutescens* gel

Figure 4.20 expresses the percentage wound closure and migration rate ($\mu m^2/h$) for *B. frutescens* gel at 24 h and 48 h exposure time periods towards HaCaT cells where the experimental values are expressed as a ratio of the untreated control group (therefore, the untreated control = 1). Microscopic images illustrating the wound areas induced by the scratch technique on HaCaT cells after 24 h and 48 h treatment with *B. frutescens* gel are depicted in Figure 4.21.

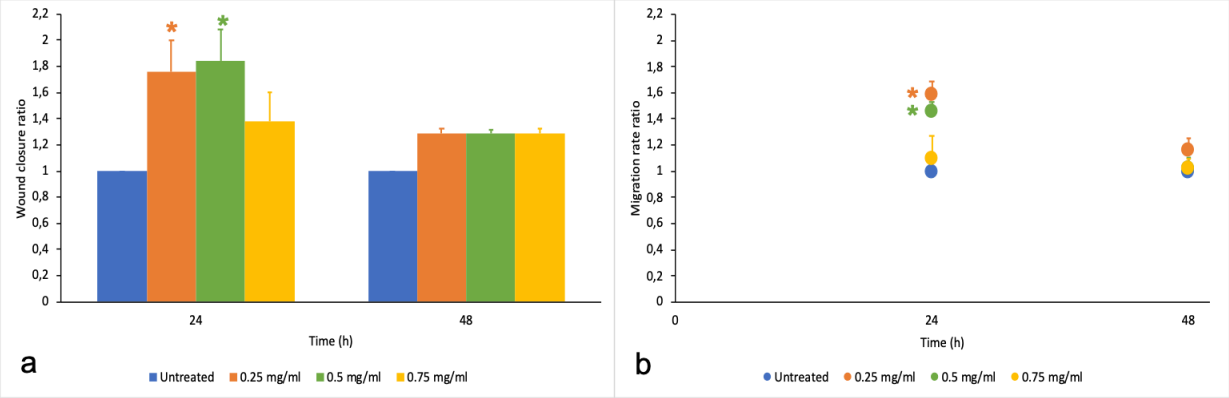


Figure 4.20: The percentage wound closure (a) and migration rate (μm^2/h) (b) results expressed as a ratio of the untreated control group of the HaCaT cells treated with *B. frutescens* gel at three concentrations over periods of 24 h and 48 h. All the data was normalised to the untreated control, which was considered as 1, as indicated on the graphs. The bars and markers represent the mean wound closure and migration rate ratios (n = 3) with error bars indicating the standard deviations. Any statistical significant differences with the untreated control are indicated on the graph as determined with Tukey's HSD (*p<0.05) and Kruskal-Wallis (**p<0.05) tests.

According to the wound closure ratio values, all the treatment concentrations of *B. frutescens* gel exhibited enhanced wound healing effects compared to that of the untreated control group. However, only the 0.25 mg/ml and 0.5 mg/ml groups exhibited ratio values of 1.76 and 1.84, respectively, at 24 h that was statistically significantly higher in comparison to the control group (Figure 4.20a). After 48 h, all the *B. frutescens* gel treatment groups almost completely closed the wound areas, exhibiting 98.25% (0.25 mg/ml), 98.42% (0.5 mg/ml) and 98.42% (0.75 mg/ml) wound closure, respectively, compared to the untreated control group that only showed 76.52% wound closure. The two lowest treatment concentrations also showed significant differences in migration rate ratios at 24 h when compared to the untreated control, exhibiting p-values of 0.000177 (0.25 mg/ml) and 0.000305 (0.5 mg/ml), respectively (Tukey's HSD test).

The *in vitro* wound healing results are in accordance with the *in vivo* wound healing potential of *B. frutescens* gel that has previously been shown by Pather *et al.* (2011:364) and Pather and Kramer (2012:523). *B. frutescens* gel was included in this study to investigate its wound healing effects when combined with other plant extracts and also to compare its wound healing effects with that of the *B. frutescens* aqueous extract.

Interestingly, the *B. frutescens* gel concentrations 0.25 mg/ml and 0.5 mg/ml showed higher wound closure ratios at 24 h (Figure 4.20a) than the *B. frutescens* aqueous extract treatment concentrations (Figure 4.18a). The aqueous extract was prepared from the whole dried plant, while the gel was removed from the inner gel part of the leaves only. The NMR spectra indicated differences in the chemical composition of the *B. frutescens* aqueous extract compared to that of the *B. frutescens* gel material. From the wound closure results, it is apparent that the *B. frutescens* gel contained phytochemicals that provided wound closure at a faster rate and to a higher extent than that of the *B. frutescens* aqueous extract material.

The migration rate escalated up to 24 h, but slowed down towards 48 h as expected due to contact inhibiton, as described before (Section 4.3.1.3).

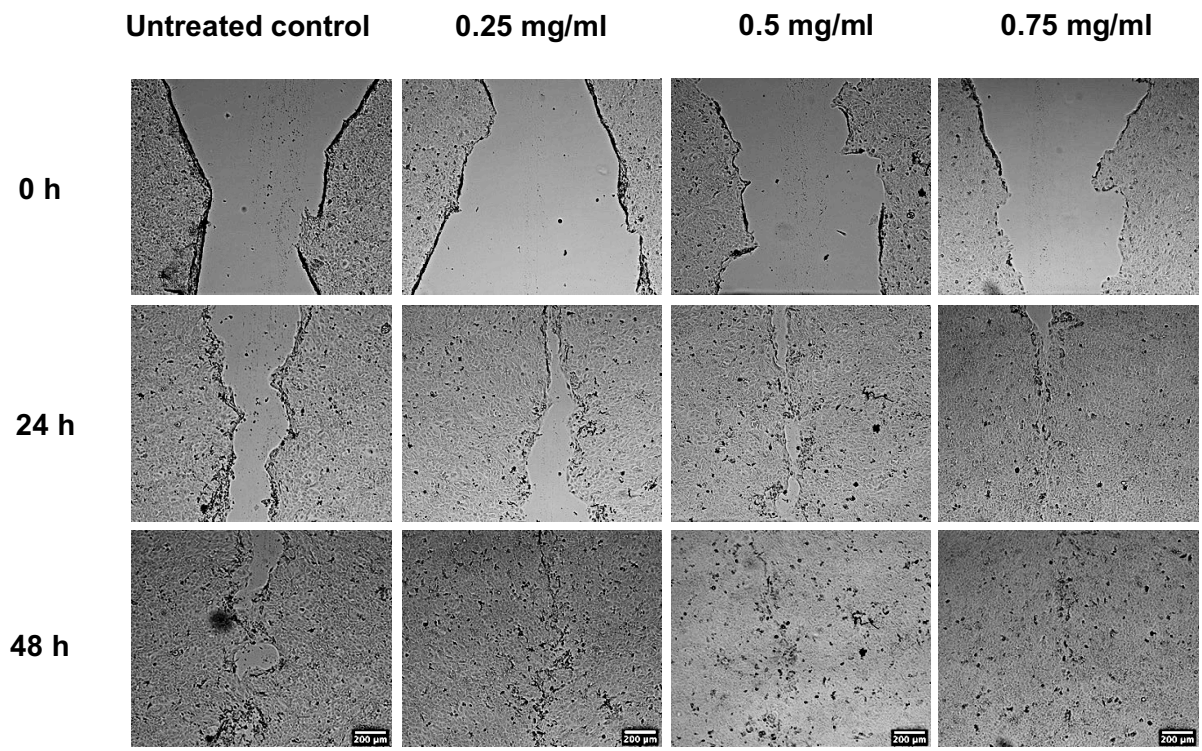
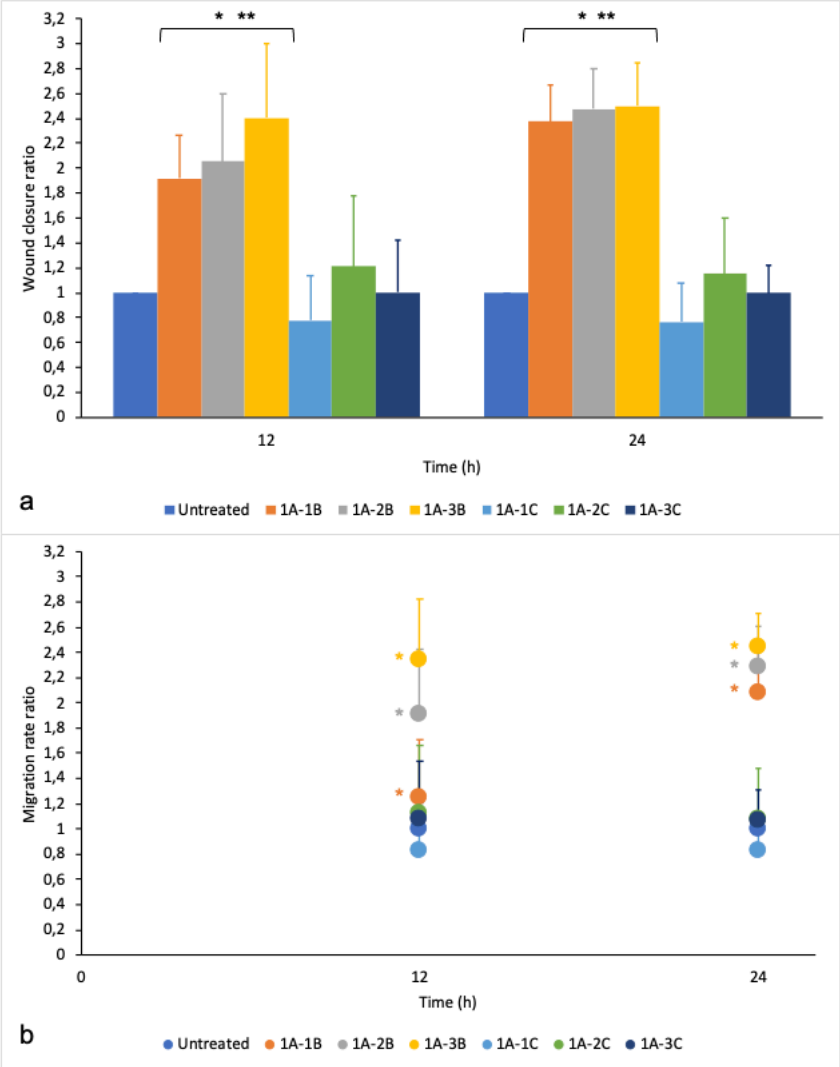


Figure 4.21: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 0.25 mg/ml, 0.5 mg/ml and 0.75 mg/ml *B. frutescens* gel for time periods of 0; 24 and 48 h. Images were captured at 10x magnification and scale bars show 200 µm.

4.3.2 Combinations of the selected plant materials

4.3.2.1 *A. vera* gel (0.25 mg/ml) combined with three treatment concentrations (0.25; 0.5 and 0.75 mg/ml) of *B. frutescens* aqueous extract and *B. frutescens* gel, respectively.

Figure 4.22 illustrates the percentage wound closure and migration rate ($\mu m^2/h$) for *A. vera* gel combined with *B. frutescens* aqueous extract and *B. frutescens* gel, respectively, at 12 h and 24 h exposure time periods towards HaCaT cells where the experimental values are expressed as a ratio of the untreated control group (therefore, the untreated control = 1).



1A = 0.25 mg/ml *A. vera* gel; 1B = 0.25 mg/ml *B. frutescens* extract; 2B = 0.50 mg/ml *B. frutescens* extract; 3B = 0.75 mg/ml *B. frutescens* extract; 1C = 0.25 mg/ml *B. frutescens* gel 2C = 0.50 mg/ml *B. frutescens* gel; 3C = 0.75 mg/ml *B. frutescens* gel

Figure 4.22: The percentage wound closure (a) and migration rate ($\mu m^2/h$) (b) results expressed as a ratio of the untreated control group of the HaCaT cells treated with *A. vera* gel combined with three concentrations of *B. frutescens* aqueous extract and *B. frutescens* gel, respectively, over periods of 12 h and 24 h. All the data was normalised to the untreated control group, which was considered as 1, as indicated on the graphs. The bars and markers represent the mean wound closure and migration rate ratios (n = 6) with error bars indicating the standard deviations. Any statistical significant differences with the untreated control are indicated on the graph as determined with Tukey's HSD (*$p<0.05$) and Kruskal-Wallis (**$p<0.05$) tests.

According to the wound closure ratio values, treatment combinations 1A-1B, 1A-2B and 1A-3B (combinations between *A. vera* gel and *B. frutescens* aqueous extract) showed significantly higher ratio values than the untreated control group, exhibiting ratio values varying between 1.92 to 2.41 and 2.38 to 2.50 at 12 h and 24 h, respectively, which indicated a more than a 2-fold increase in wound closure and migration rate at 24 h compared to the untreated control. Treatment combinations 1A-1B, 1A-2B and 1A-3B also showed significant differences in migration rate ratios at 12 h and 24 h when compared to the untreated control (Tukey's HSD test). None of the *A. vera* gel and *B. frutescens* gel (1A-1C, 1A-2C and 1A-3C) combinations were statistically significantly different when compared to the untreated control group.

As previously mentioned in Section 4.3.1.3, *B. frutescens* aqueous extract demonstrated wound closure ratios varying from 1.5 to 1.7 at 24 h, while combinations of this plant material with 0.25 mg/ml *A. vera* gel induced more than a 2-fold increase in wound closure and migration rate at 24 h treatment periods when compared to the untreated control. Interestingly, of all the *A. vera* gel treatment concentrations tested, 0.25 mg/ml also exhibited the highest wound closure and migration rate ratio at 24 h and 48 h treatment periods. Microscopic images illustrating the wound areas induced by the scratch technique on HaCaT cells after 12 h and 24 h exposure to treatment combinations 1A-1B, 1A-2B, 1A-3B, 1A-1C, 1A-2C and 1A-3C are depicted in Figure 4.23a and 4.23b.

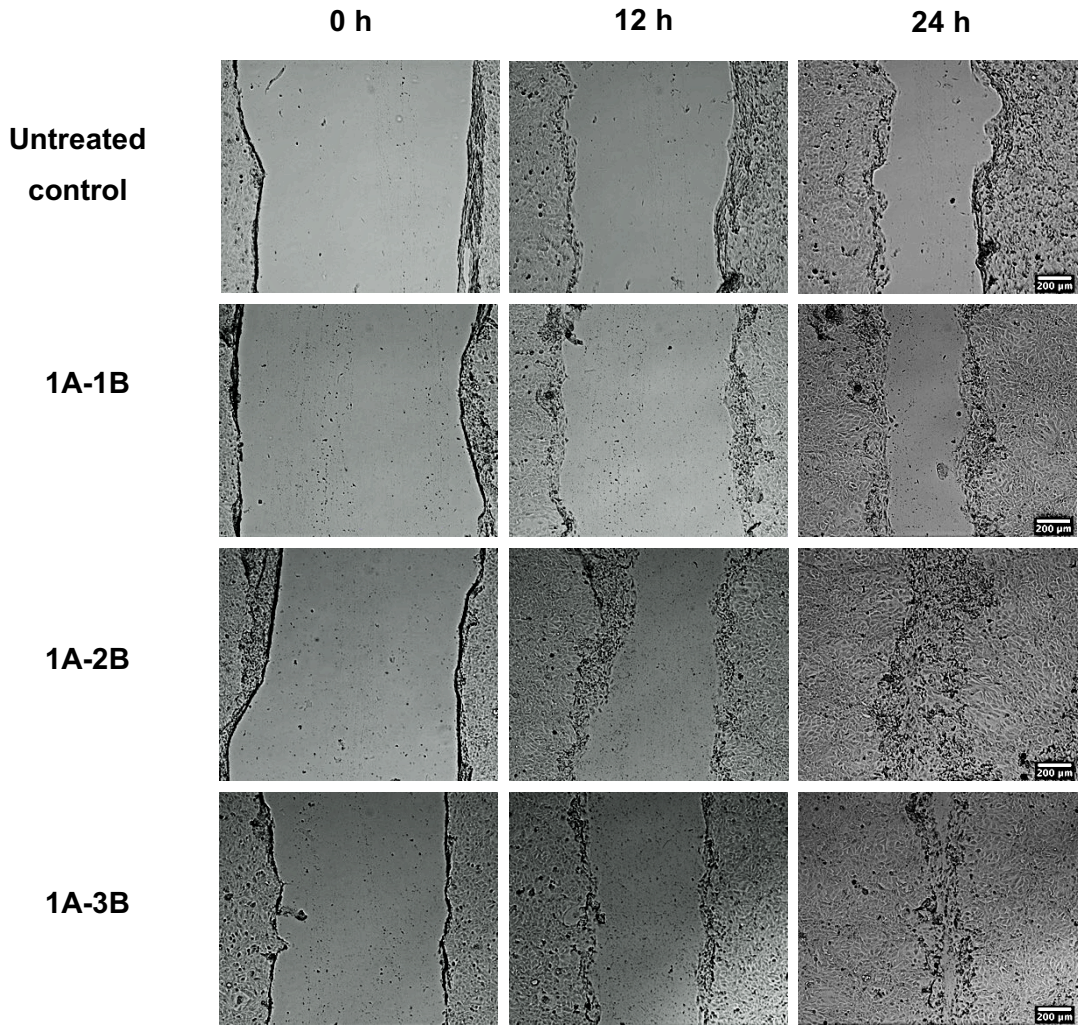


Figure 4.23a: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to treatment combinations 1A-1B, 1A-2B and 1A-3B for time periods of 0; 12 and 24 h. Images were captured at 10 x magnification and scale bars show 200 µm.

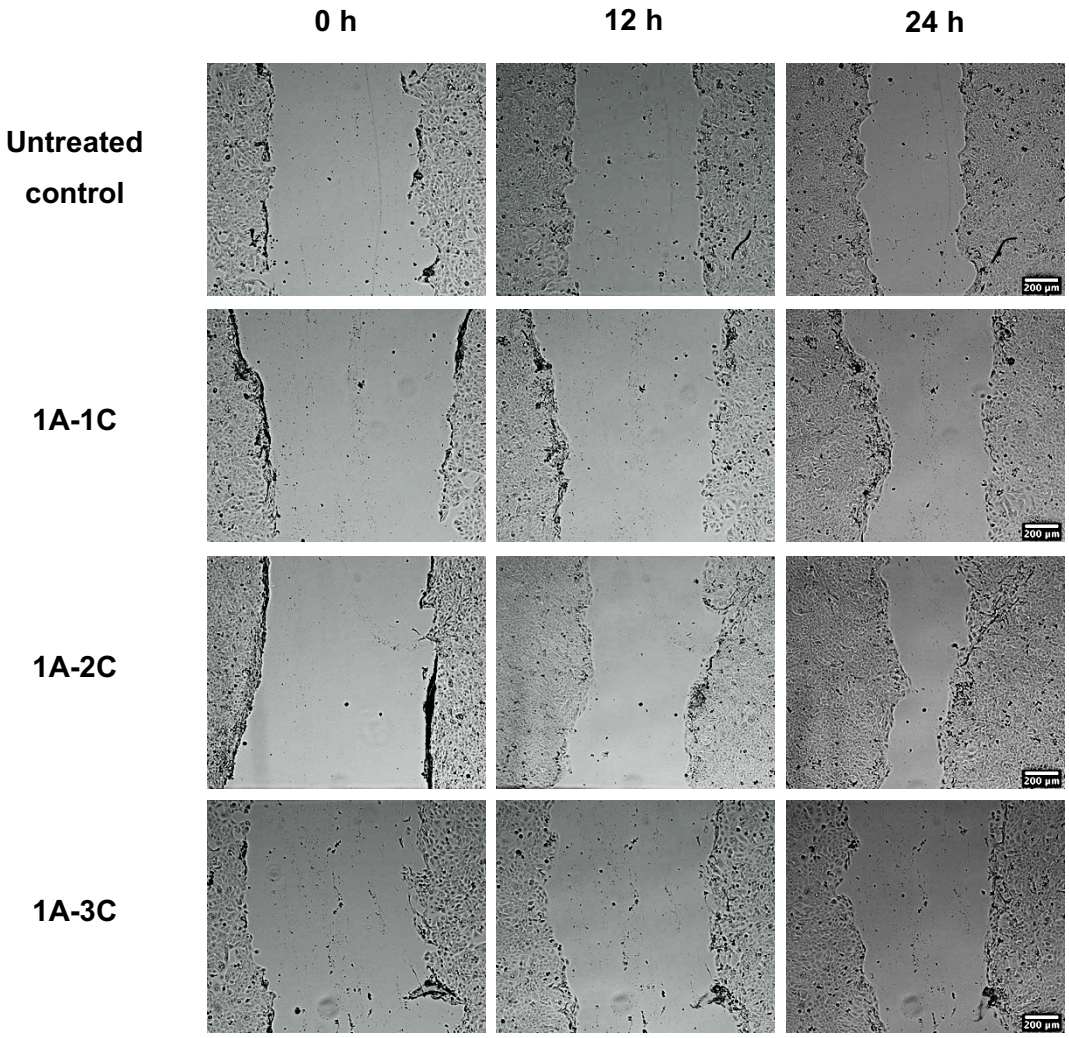
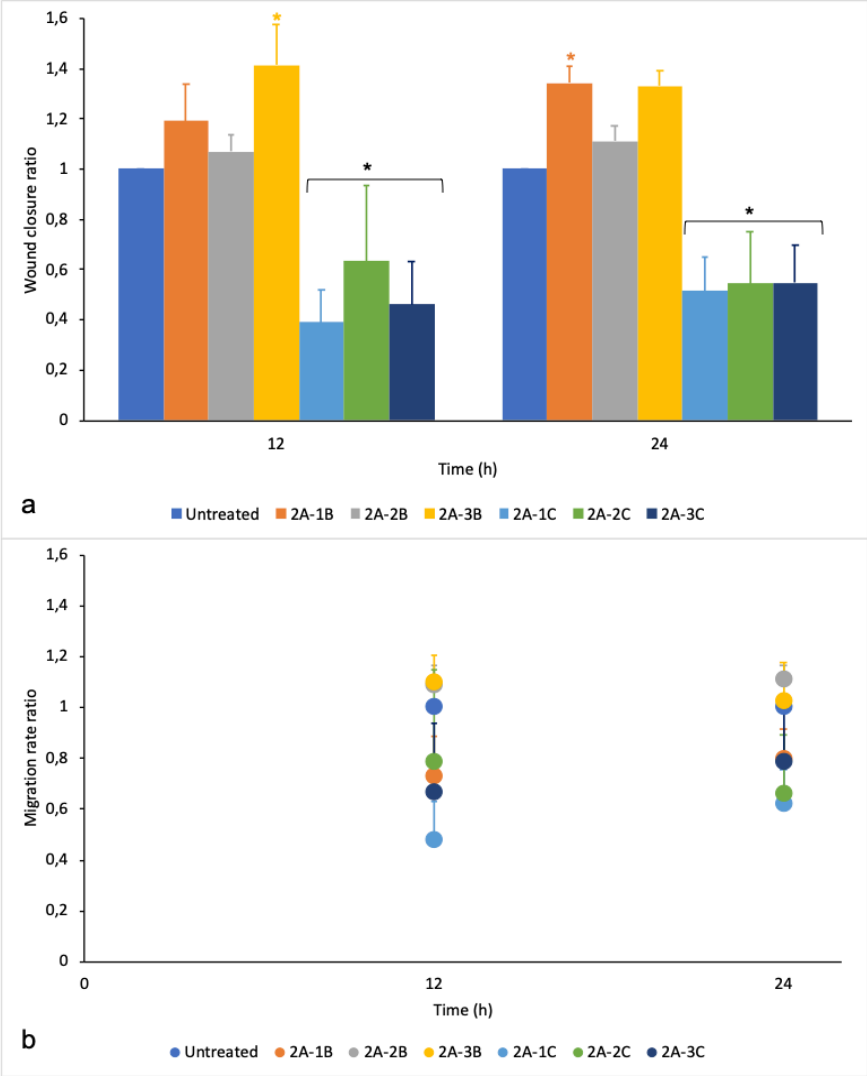


Figure 23b: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to treatment combinations 1A-1C, 1A-2C and 1A-3C for time periods of 0; 12 and 24 h. Images were captured at 10 x magnification and scale bars show 200 µm.

4.3.2.2 *A. vera* gel (0.5 mg/ml) combined with three treatment concentrations (0.25; 0.5 and 0.75 mg/ml) of *B. frutescens* aqueous extract and *B. frutescens* gel, respectively.

Figure 4.24 expresses the percentage wound closure and migration rate ($\mu m^2/h$) for combinations of *A. vera* gel with three concentrations of *B. frutescens* aqueous extract and *B. frutescens* gel, respectively, at 12 h and 24 h exposure time periods towards HaCaT cells where the experimental values are expressed as a ratio of the untreated control group (therefore, the untreated control = 1).



2A = 0.5 mg/ml *A. vera* gel; 1B = 0.25 mg/ml *B. frutescens* extract; 2B = 0.50 mg/ml *B. frutescens* extract; 3B = 0.75 mg/ml *B. frutescens* extract; 1C = 0.25 mg/ml *B. frutescens* gel 2C = 0.50 mg/ml *B. frutescens* gel; 3C = 0.75 mg/ml *B. frutescens* gel.

Figure 4.24: The percentage wound closure (a) and migration rate ($\mu m^2/h$) (b) results expressed as a ratio of the untreated control group of the HaCaT cells treated with *A. vera* gel combined with three concentrations of *B. frutescens* aqueous extract and *B. frutescens* gel, respectively, over periods of 12 h and 24 h. All the data was normalised to the untreated control group, which was considered as 1, as indicated on the graphs. The bars and markers represent the mean wound closure and migration rate ratios (n = 6) with error bars indicating the standard deviations. Any statistical significant differences with the untreated control are indicated on the graph as determined with Tukey's HSD (*$p<0.05$) and Kruskal-Wallis (**$p<0.05$) tests.

The HaCaT cells exposed to combinations 2A-1B; 2A-2B and 2A-3B showed an increase in wound closure ratio at 12 h and 24 h treatment periods when compared to the untreated control. Combination 2A-3B and 2A-1B exhibited a statistically significant increase in wound closure at 12 h and 24 h, respectively, relative to the untreated control. Unfortunately, the *A. vera* gel and *B. frutescens* gel concentration combinations (2A-1C; 2A-2C and 2A-3C) tested showed a significant decrease in wound closure and migration rate ratios when compared to that of the untreated control group (Tukey's HSD test). Only combinations 2A-2B and 2A-3B showed an increase in migration rate ratio, but not significantly. Microscopic images illustrating the wound areas induced by the scratch technique on HaCaT cells after 12 h and 24 h exposure to treatment combinations 2A-1B, 2A-2B, 2A-3B, 2A-1C, 2A-2C and 2A-3C are depicted in Figure 4.25a and 4.25b.

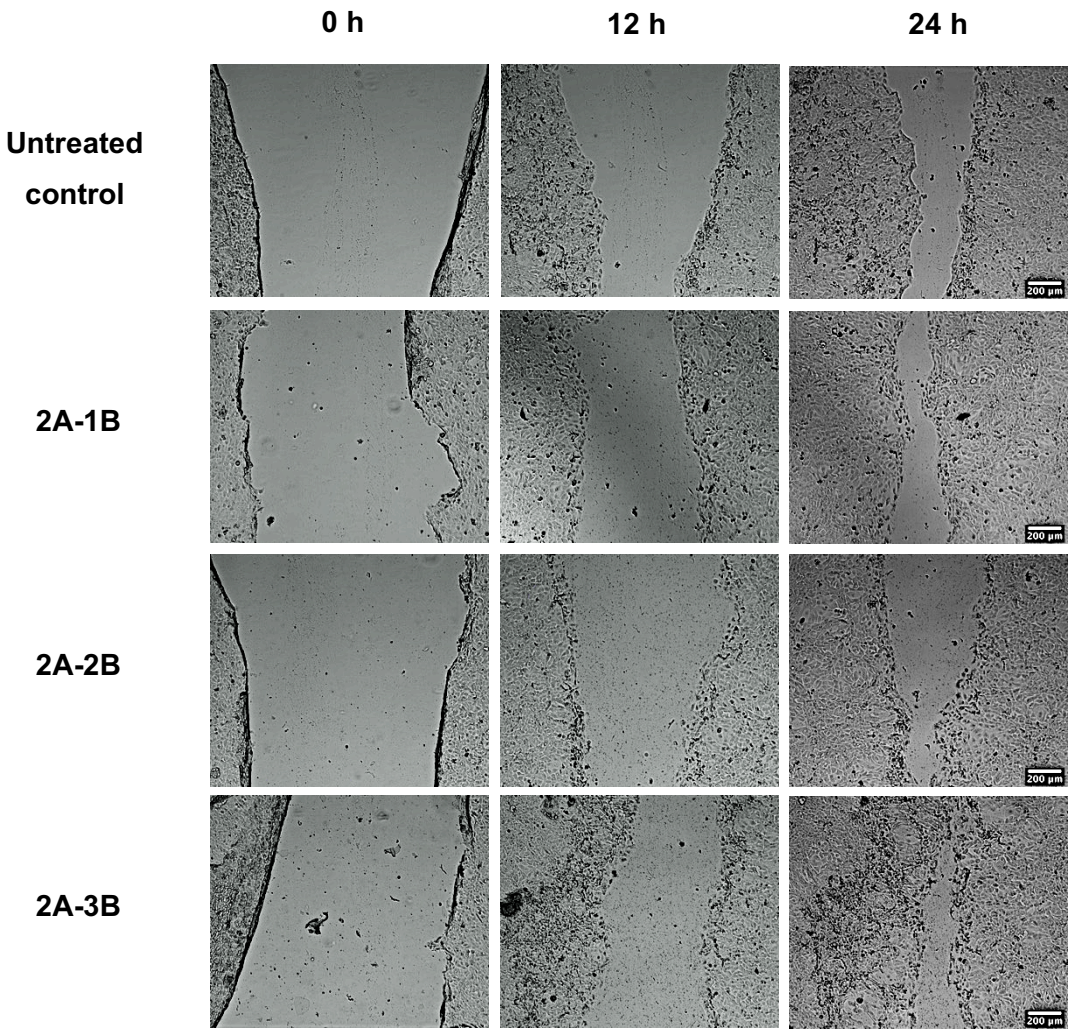


Figure 4.25a: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 2A-1B, 2A-2B and 2A-3B for time periods of 0; 12 and 24 h. Images were captured at 10 x magnification and scale bars show 200 µm.

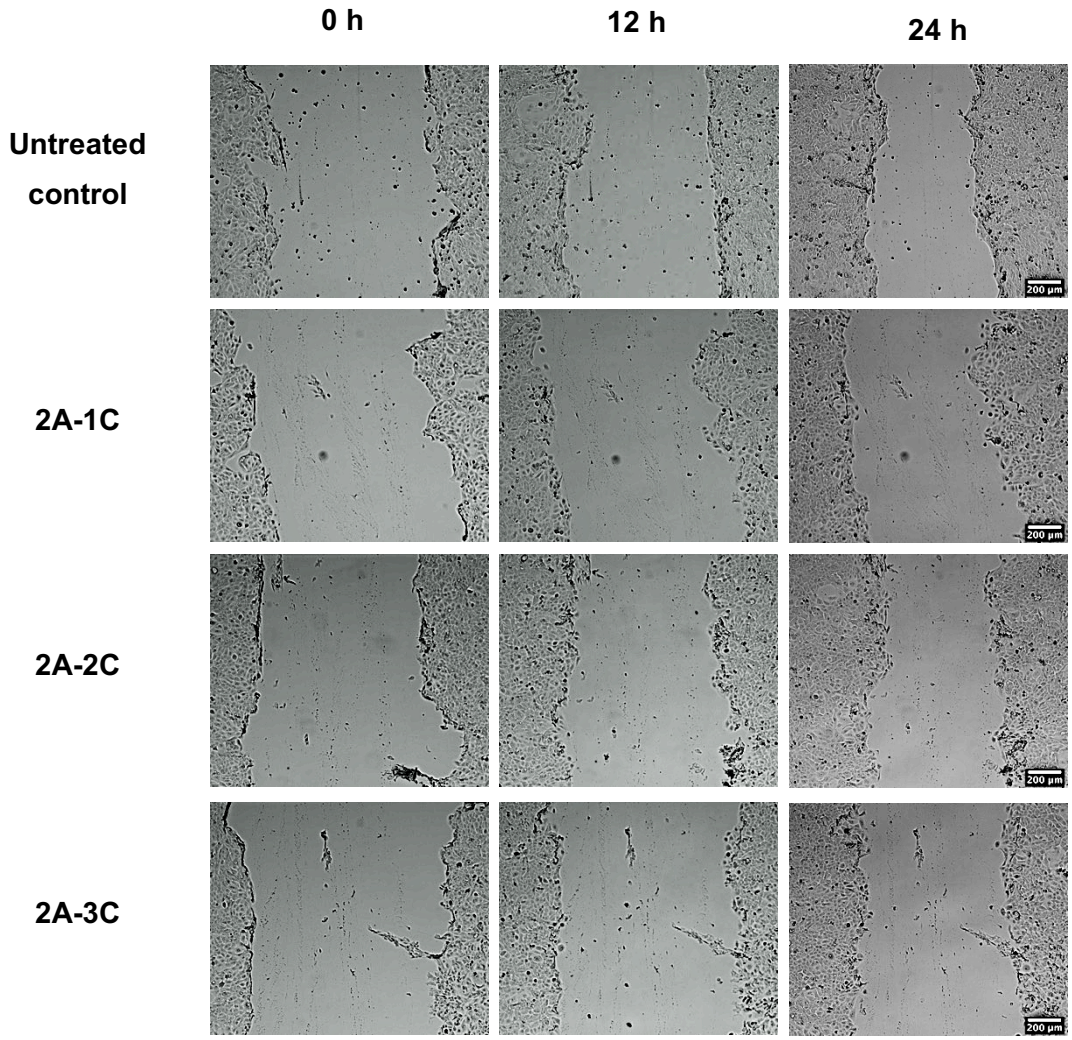
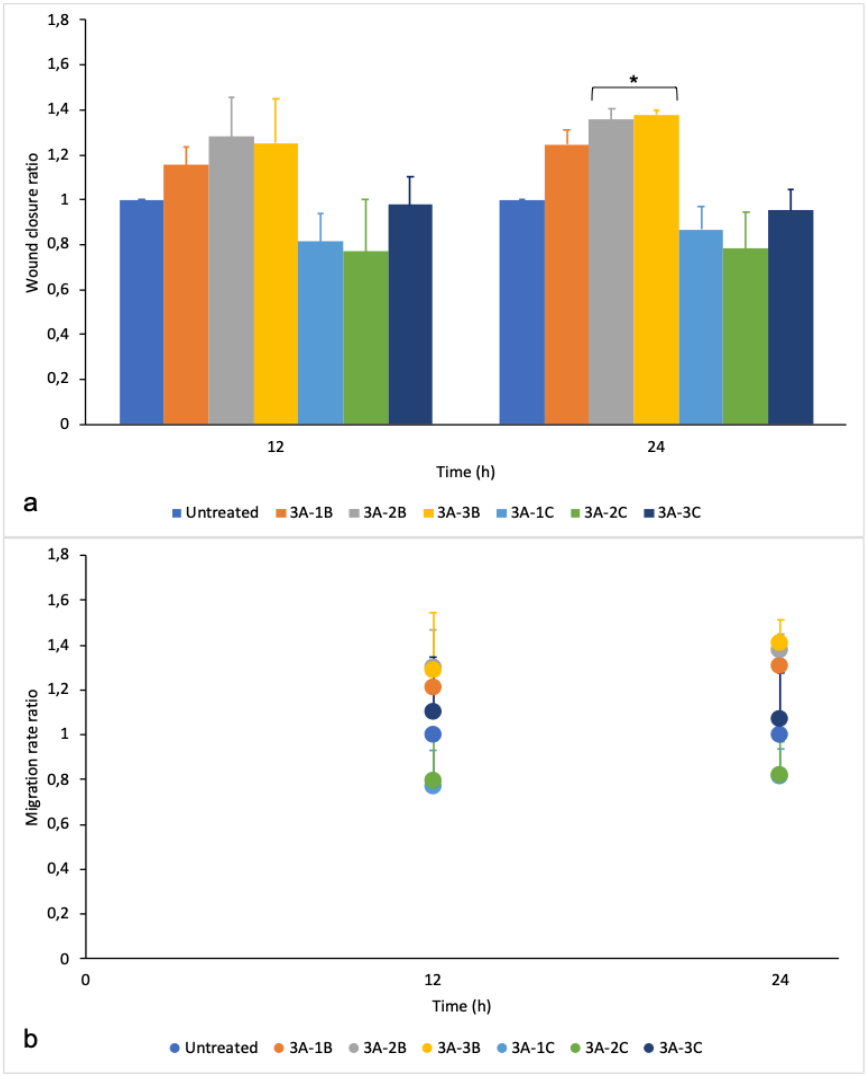


Figure 25b: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 2A-1C, 2A-2C and 2A-3C for time periods of 0; 12 and 24 h. Images were captured at 10 x magnification and scale bars show 200 µm.

4.3.2.3 *A. vera* gel (0.75 mg/ml) combined with three treatment concentrations (0.25; 0.5 and 0.75 mg/ml) of *B. frutescens* aqueous extract and *B. frutescens* gel, respectively.

Figure 4.26 expresses the percentage wound closure and migration rate ($\mu m^2/h$) for combinations *of A. vera* gel (0.75 mg/ml) with three concentrations of *B. frutescens* aqueous extract and *B. frutescens* gel, respectively, at 12 h and 24 h exposure time periods towards HaCaT cells where the experimental values are expressed as a ratio of the untreated control group (therefore, the untreated control = 1).



3A = 0.75 mg/ml *A. vera* gel; 1B = 0.25 mg/ml *B. frutescens* extract; 2B = 0.50 mg/ml *B. frutescens* extract; 3B = 0.75 mg/ml *B. frutescens* extract; 1C = 0.25 mg/ml *B. frutescens* gel 2C = 0.50 mg/ml *B. frutescens* gel; 3C = 0.75 mg/ml *B. frutescens* gel

Figure 4.26: The percentage wound closure (a) and migration rate ($\mu m^2/h$) (b) results expressed as a ratio of the untreated control group of the HaCaT cells treated with *A. vera* gel combined with three concentrations of *B. frutescens* aqueous extract and *B. frutescens* gel, respectively, to the untreated control group over periods of 12 h and 24 h. All the data was normalised to the untreated control, which was considered as 1, as indicated on the graphs. The bars and markers represent the mean wound closure and migration rate ratios (n = 6) with error bars indicating the standard deviations. Any statistical significant differences with the untreated control are indicated on the graph as determined with Tukey's HSD (*p<0.05) and Kruskal-Wallis (**p<0.05) tests.

According to the wound closure ratio values, treatment combinations 3A-1B; 3A-2B and 3A-3B exhibited enhanced wound healing effects compared to that of the untreated control group, but only combinations 3A-2B and 3A-3B showed statistically significantly higher values at 24 h, relative to the untreated control group. Treatment combinations 3A-1B; 3A-2B and 3A-3B also showed an increase in migration rate ratio, relative to the untreated control. None of the *A. vera* gel (0.75 mg/ml) and *B. frutescens* gel (0.25; 0.5 and 0.75 mg/ml) treatment combination demonstrated an increase in wound closure and migration rate, except for treatment combination 3A-3C, which demonstrated an increase in migration rate ratio.

As mentioned previously in Section 4.3.1.3 and 4.3.1.4, *B. frutescens* gel demonstrated a higher wound healing capability at 24 h compared to *B. frutescens* aqueous extract when tested individually. However, the same favourable wound healing results were not obtained with combinations of *B. frutescens* gel and *A. vera* gel. Interestingly, combinations of *A. vera* gel with *B. frutescens* aqueous extract showed enhanced wound healing compared to the individually tested plant materials. Microscopic images illustrating the wound areas induced by the scratch technique on HaCaT cells after 12 h and 24 h exposure to treatment combinations 3A-1B, 3A-2B, 3A-3B, 3A-1C, 3A-2C and 3A-3C are depicted in Figure 4.27a and 4.27b.

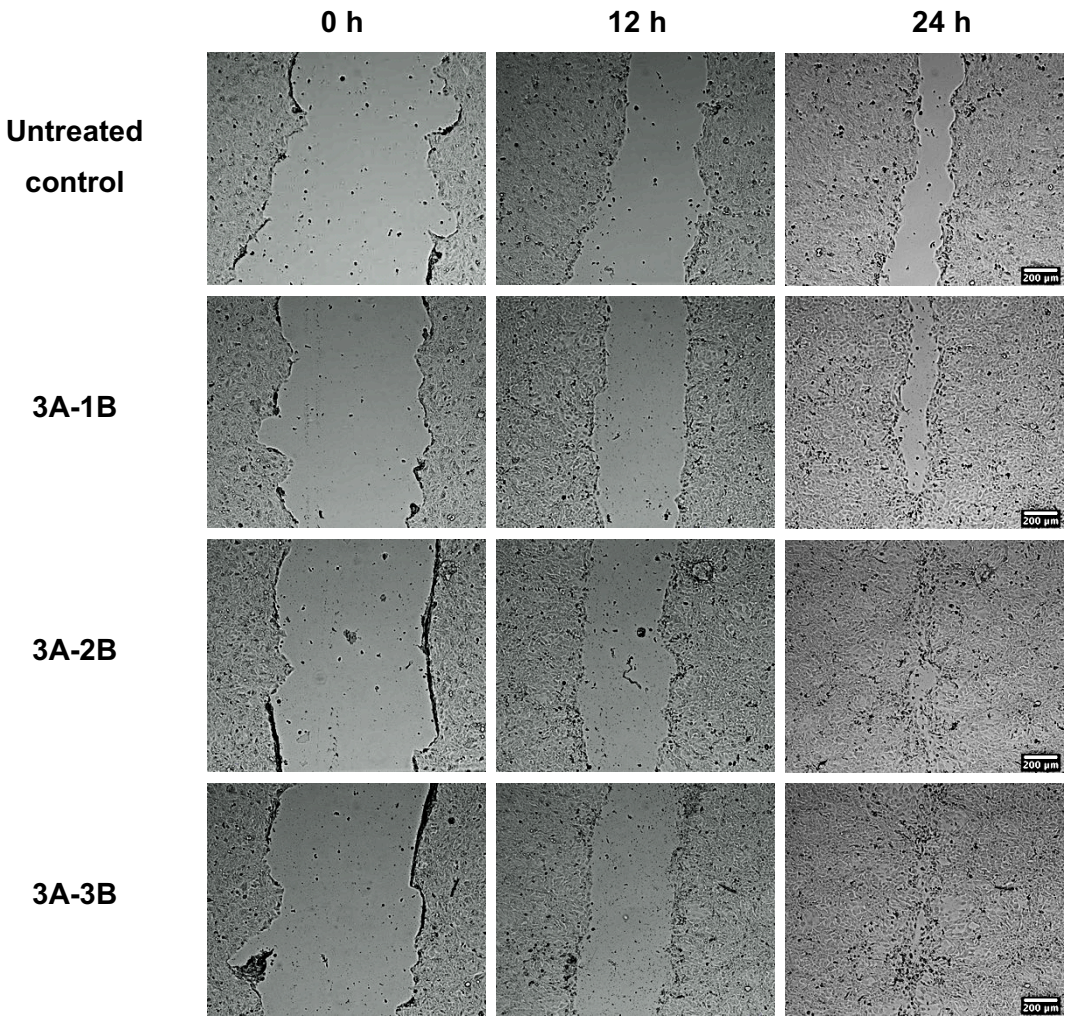


Figure 4.27a: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 3A-1B, 3A-2B and 3A-3B for time periods of 0; 12 and 24 h. Images were captured at 10 x magnification and scale bars show 200 µm.

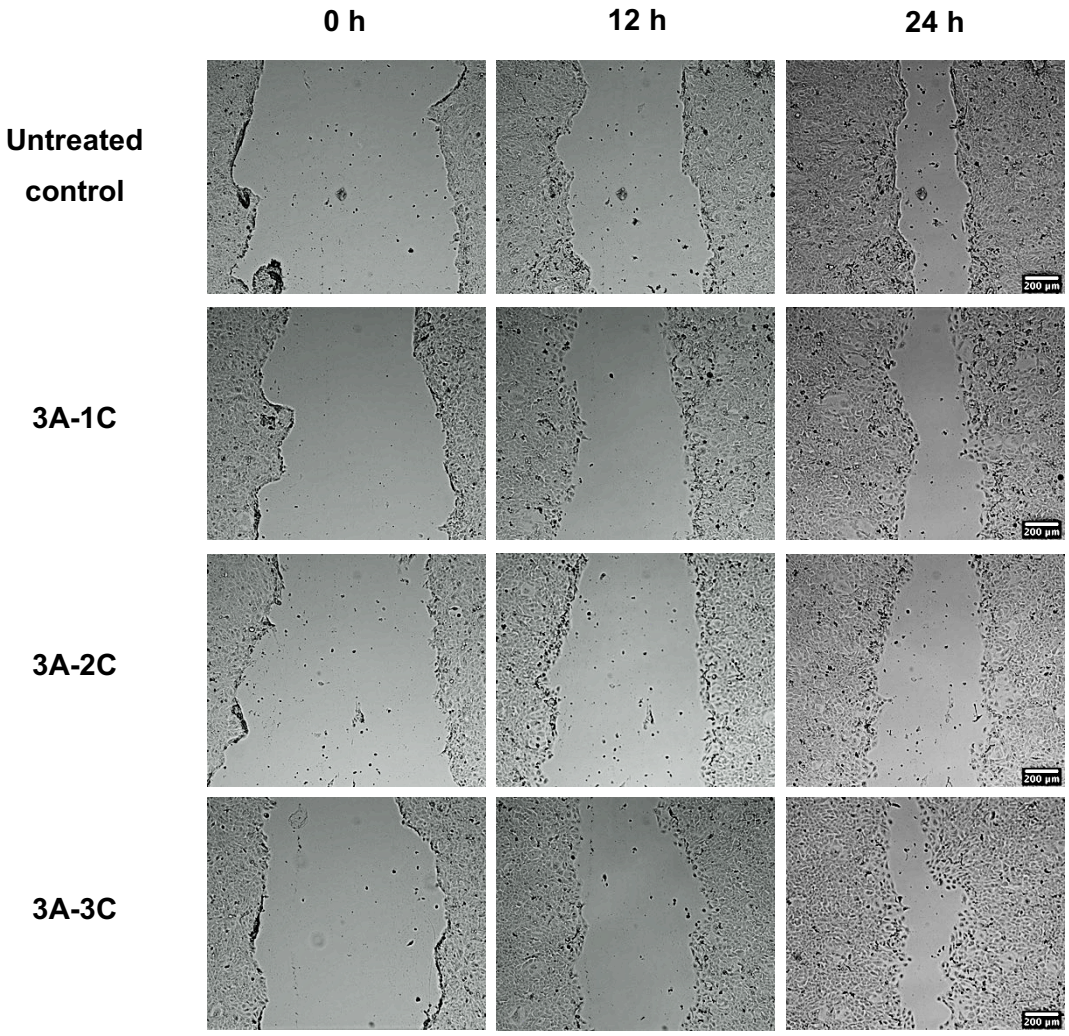
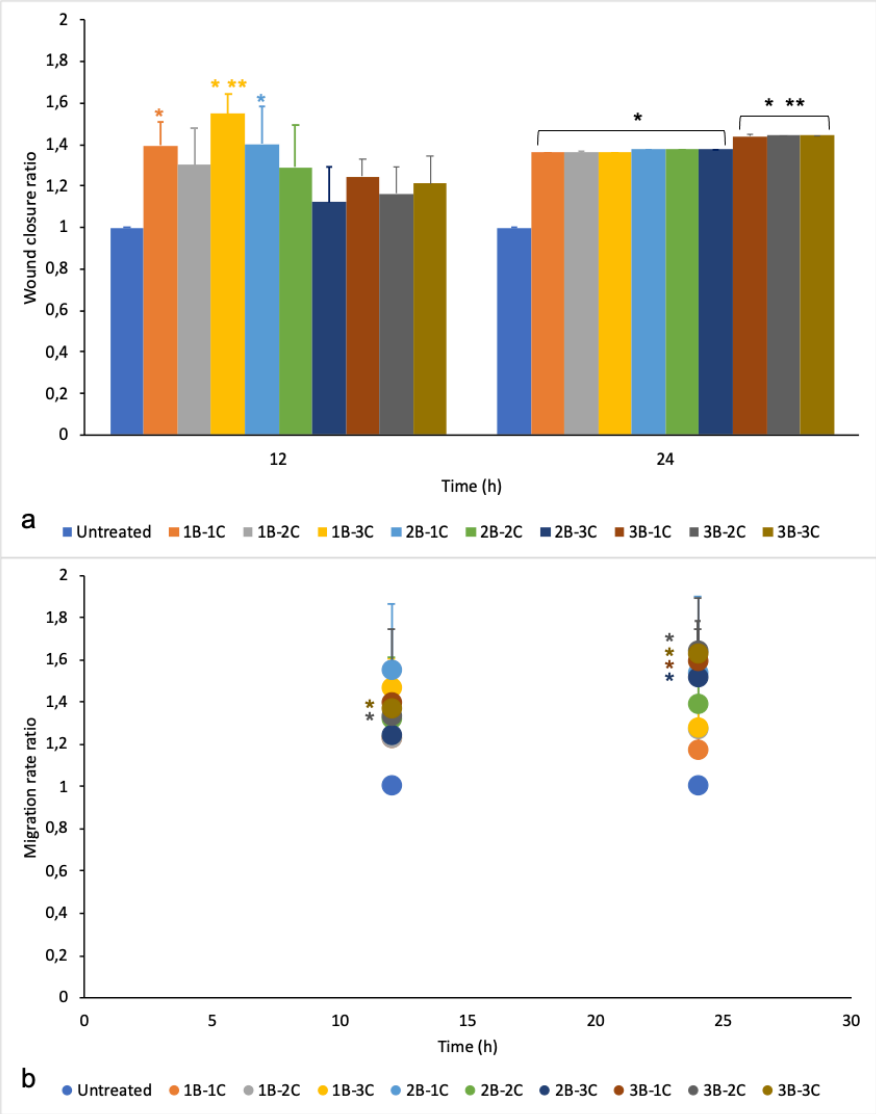


Figure 27b: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 3A-1C, 3A-2C and 3A-3C for time periods of 0; 12 and 24 h. Images were captured at 10 x magnification and scale bars show 200 µm.

4.3.2.4 Combinations of *B. frutescens* aqueous extract and *B. frutescens* gel

Figure 4.28 expresses the percentage wound closure and migration rate ($\mu m^2/h$) for different concentration combinations of *B. frutescens* aqueous extract and *B. frutescens* gel, respectively, at 12 h and 24 h exposure time periods towards HaCaT cells where the experimental values are expressed as a ratio of the untreated control group (therefore, the untreated control = 1).



1B = 0.25 mg/ml *B. frutescens* extract; 2B = 0.50 mg/ml *B. frutescens* extract; 3B = 0.75 mg/ml *B. frutescens* extract; 1C = 0.25 mg/ml *B. frutescens* gel 2C = 0.50 mg/ml *B. frutescens* gel; 3C = 0.75 mg/ml *B. frutescens* gel.

Figure 4.28: The percentage wound closure (a) and migration rate ($\mu m^2/h$) (b) results expressed as a ratio of the untreated control group of the HaCaT cells treated with different concentrations and combinations of *B. frutescens* gel and *B. frutescens* aqueous extract, respectively, over periods of 12 h and 24 h. All the data was normalised to the untreated control group, which was considered as 1, as indicated on the graphs. The bars and markers represent the mean wound closure and migration rate ratios (n = 6) with error bars indicating the standard deviations. Any statistical significant differences with the untreated control are indicated on the graph as determined with Tukey's HSD (*p<0.05) and Kruskal-Wallis (**p<0.05) tests.

All the *B. frutescens* extract and *B. frutescens* gel treatment combinations showed an increase in wound closure ratio compared to that of the untreated control. Combinations 1B-1C, 1B-3C and 2B-1C showed a significant increase in wound closure at 12 h, while all the treatment concentrations were statistically significantly higher at 24 h compared to the untreated control (Figure 4.28a). At 24 h, the wounds were almost completely closed in all the experimental groups, unlike the untreated control group. According to the migration rate ratio values, all the treatment concentrations showed an increase in migration rate. Treatment combinations 3B-2C and 3B-3C showed a significant increase in migration rate at 12 h, whilst at 24 h combinations 2B-3C, 3B-1C, 3B-2C and 3B-3C resulted in a significant increase in migration rate. Microscopic images illustrating the wound areas induced by the scratch technique on HaCaT cells after 12 h and 24 h exposure to treatment combinations of *B. frutescens* aqueous extract and *B. frutescens* gel are depicted in Figure 4.29a, 4.29b and 4.29c.

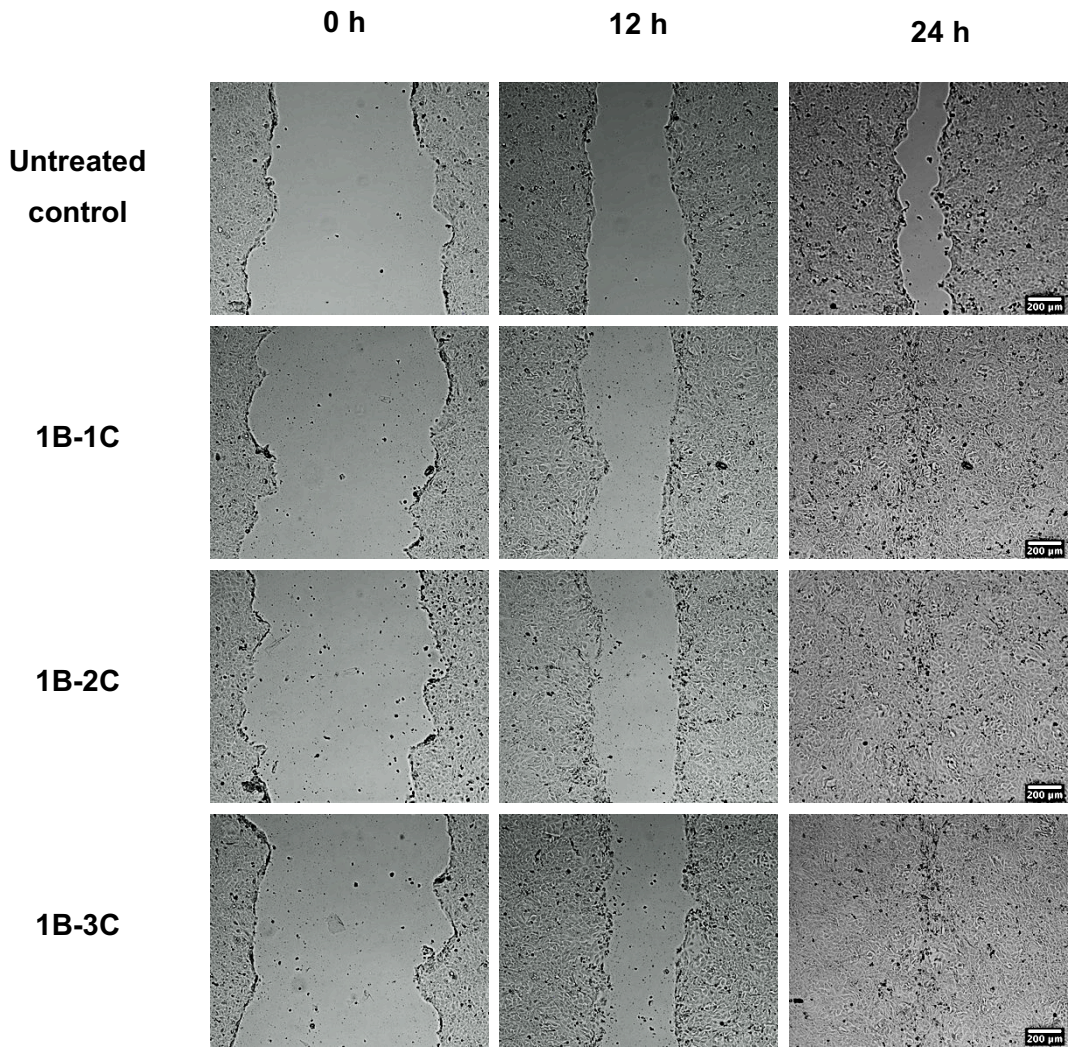


Figure 4.29a: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 1B-1C, 1B-2C and 1B-3C for time periods of 0; 12 and 24 h. Images were captured at 10 x magnification and scale bars show 200 µm.

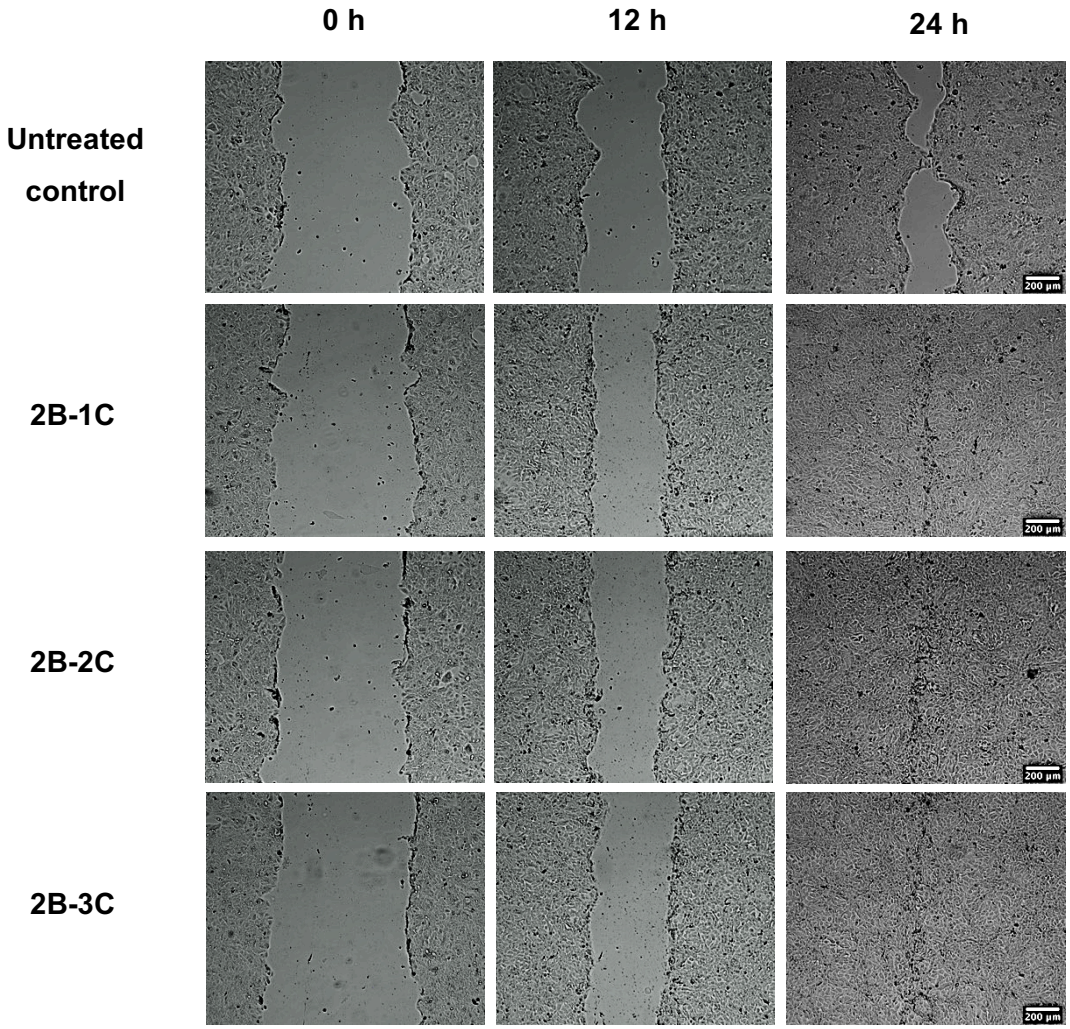


Figure 4.29b: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 2B-1C, 2B-2C and 2B-3C for time periods of 0; 12 and 24 h. Images were captured at 10 x magnification and scale bars show 200 µm. Images were captured at 10 x magnification and scale bars show 200 µm.

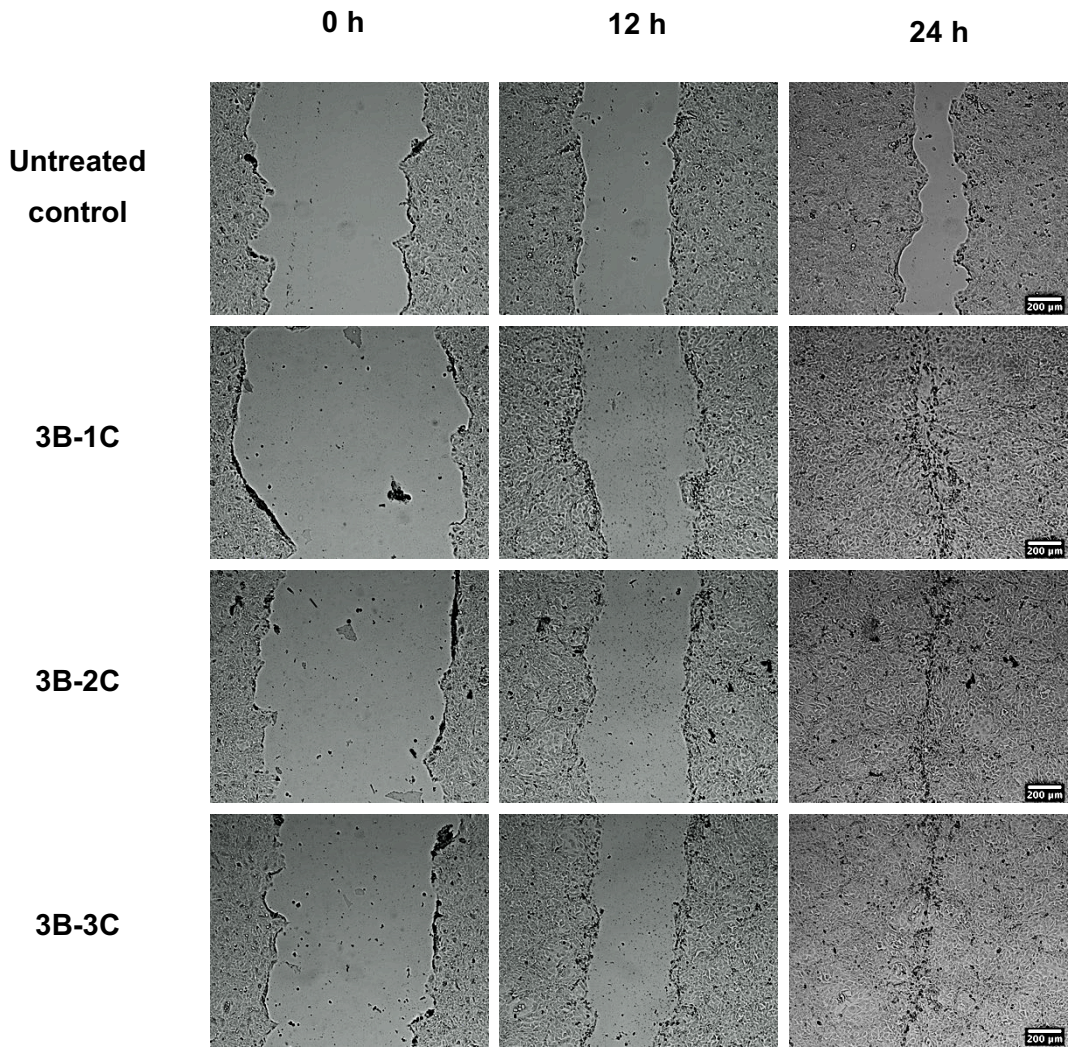


Figure 4.29c: Microscopic images of wound areas induced by the scratch technique on HaCaT cells. The microscopic images display the wound areas in the untreated control and the wound areas exposed to 3B-1C, 3B-2C and 3B-3C for time periods of 0; 12 and 24 h. Images were captured at 10 x magnification and scale bars show 200 µm

4.4 Conclusion

Both MTT and NR cytotoxicity assays were performed to establish if the individual plant materials presented cytotoxic effects on HaCaT and 84BR cells. The MTT cytotoxicity assay showed a concentration-dependent decrease in cell viability for both skin-derived cell lines after exposure to *A. vera* gel and *C. longa* extract (curcumin). The NR cytotoxicity assay also showed the same concentration-dependent decrease as with the MTT assay, however, a less toxic effect was observed with the 84BR cell line exposed to *A. vera* gel. Strong cytotoxic effects (i.e. <40% cell viability) were observed at higher treatment concentrations of *C. longa* extract (curcumin) (López-García *et al*., 2014:44). The results obtained from the NR cytotoxicity assay showed an increase in cell viability at higher treatment concentrations after exposure to *B. frutescens* aqueous extract. Similar results were observed with the MTT assay for the 84BR cells, however, the HaCaT cells only demonstrated no cytotoxicity after a 48h exposure period to *B. frutescens* aqueous extract. The MTT and NR cytotoxicity assays indicated that none of the *B. frutescens* gel treatment concentrations demonstrated cytotoxic effects on HaCaT or 84BR cells.

A. vera gel showed wound healing effects/characteristics by higher wound closure values as compared to the untreated control, but it was not statistically significant. Unfortunately, none of the *C. longa* extract (curcumin) concentrations tested showed *in vitro* wound healing properties, which is in contrast to findings from previous *in vivo* wound healing studies (Sidhu *et al*., 1998:176; Sidhu *et al.,* 1999:372; Panchatcharam *et al.,* 2006:9; Gadekar *et al.,* 2012:229; Ghosh & Gaba, 2013:767; Akbik *et al.,* 2014:2; Yen *et al.,* 2018:612). *B. frutescens* extract exhibited significant wound closure after 24 h at all three concentrations, while only the two highest concentrations showed a significant increase in migration rate when compared to the untreated control. All three treatment concentrations of *B. frutescens* gel also displayed increased wound healing effects, but only the two lowest concentrations showed a significant increase in wound closure and migration rate after the 24 h treatment period.

From the results obtained in this study, it is clear that *A. vera* gel, *B. frutescens* aqueous extract and *B. frutescens* gel proved to have wound healing capabilities when tested individually. Therefore, only these three medicinal plant extracts were used to create different concentration combinations and determine its *in vitro* wound healing potential by means of the scratch assay on HaCaT cell monolayers.

As previously mentioned, *B. frutescens* gel exhibited better wound healing effects than *B. frutescens* extract when tested individually. Interestingly, combinations of these plant materials with *A. vera* gel showed different results. All the *B. frutescens* extract and *A. vera* gel treatment combinations showed higher wound healing properties. *B. frutescens* extract presented wound

closure and migration rate ratios varying from 1.5 to 1.7 and 1.38 to 1.66, respectively, but combined with *A. vera* gel more than a 2-fold increase in wound closure and migration rate was obtained at 24 h. Interestingly, the lowest concentrations of *A. vera* gel (0.25 mg/ml) also showed the best wound healing properties when tested individually. *B. frutescens* aqueous extract combined with *A. vera* gel enhanced wound healing by increasing the wound closure values as well as the migration rate of the cells into the wound field. It is clear from the results that combinations of *A. vera* gel and *B. frutescens* gel had a negative effect on the wound healing process and migratory activity of the cells when compared to the individual gel materials. All the *B. frutescens* aqueous extract and *B. frutescens* gel combinations demonstrated an increased tendency to enhance wound closure, as well as the migratory activity of the cells.

Combinations of *B. frutescens* extract with *B. frutescens* gel and *A. vera* gel showed enhanced wound healing capabilities as compared to the individual materials. On the other hand, combinations of *A. vera* gel with *B. frutescens* gel demonstrated less favourable wound healing results as compared to the individual materials.

4.5 References

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CHAPTER 5 CONCLUDING REMARKS AND FUTURE RECOMMENDATIONS

5.1 Concluding remarks

Re-epithelialisation forms a significant part in the wound healing process, which involves the migration and proliferation of keratinocytes to cover a wound gap with new epithelium (Santoro & Gaudino, 2004:274; Rousselle *et al.,* 2019:345). The epidermis, which forms the outermost layer of the skin, consists mostly of keratinocytes (Rousselle *et al.,* 2019:345). Skin wounds that are unable to re-epithelialise will fail to proceed through the normal phases of wound healing and the wound will re-occur to form chronic wounds (Velnar *et al.,* 2009:1529; Rousselle *et al.,* 2019:348). For decades, herbs and medicinal plants have been used for the treatment and management of wounds and their use is still highly valued and very popular in our modern society (Sabale *et al*., 2012:145-148).

A. vera is the most mercantile Aloe species and has been widely used in traditional medicine practices for centuries (Steenkamp & Stewart, 2007:411; Hamman, 2008:1600). The wound healing as well as other therapeutic effects of materials acquired from *A. vera*, which has been thoroughly researched over the past few years, has been disclosed in literature (Khorasani *et al.* 2009:587; Oryan *et al*., 2010:509; Shahzad & Ahmed, 2013:225; Hashemi *et al*., 2015:3; Fox *et al.,* 2017:7). *C. longa* extract (curcumin), colloquially known as turmeric, has been further employed for its medicinal properties which includes anti-inflammatory, antioxidant, antimicrobial and anti-cancer effects (Maheshwari *et al*., 2006:2082). Research that includes many *in vivo* and *in vitro* studies on the wound healing properties of curcumin, the main curcuminoid in turmeric, has been performed. The wound healing properties of curcumin has been identified in distinct phases of the complex, multi-faceted wound healing process (Sidhu *et al*., 1998:176; Sidhu *et al.,* 1999:372; Panchatcharam *et al.,* 2006:91; Gadekar *et al.,* 2012:229; Ghosh & Gaba, 2013:767; Akbik *et al.,* 2014:2-6; Yen *et al.,* 2018:612).

B. frutescens is indigenous to South Africa and has been extensively used as a traditional wound healing agent (Phakamani, 2008:166; Pather *et al*., 2011:364; Pather and Kramer 2012:523). However, limited information is available on its chemical properties, cytotoxicity and wound healing properties, justifying further investigation. Both *B. frutescens* gel and *B. frutescens* aqueous extract were included in this study. The aqueous extract was prepared from the whole dried plant, while the gel was removed from the inner gel part of the leaves only. The nuclear magnetic resonance (both ^{1}H-NMR and ^{13}C-NMR) spectra were used as a qualitative method to obtain the chemical fingerprint of these plant materials. The phytochemical contents of *B.*

frutescens gel and aqueous extract showed some similarities, but different chemical components were also present in each extracted fraction as evident from the peaks on the NMR spectra.

The MTT and NR cytotoxicity assays were performed to evaluate the percentage cell viability of both HaCaT and 84BR cells after 24 h and 48 h exposure to *A. vera* gel*, C. longa* extract (curcumin), *B. frutescens* aqueous extract and *B. frutescens* gel. These cytotoxicity assays were utilised as preliminary screening to establish the treatment concentrations of the individual plant materials that were safe to use on these human skin cells for wound healing effects.

This study investigated the *in vitro* wound healing potential of *A. vera gel, C. longa* extract (curcumin), *B. frutescens* aqueous extract and *B. frutescens* gel individually at three treatment concentrations. In addition, this study evaluated different combinations of the selected individual plant materials to determine if a more enhanced wound healing effect could be observed as compared to their individual performances. The wound healing properties was evaluated by means of a scratch assay in cultured HaCaT cell monolayers.

A. vera gel*, B. frutescens* aqueous extract and *B. frutescens* gel exhibited pronounced wound healing effects when tested individually as compared to that of the control group. *A. vera* gel showed wound healing effects when compared to the untreated control, but it was not statistically significant. *B. frutescens* aqueous extract and *B. frutescens* gel showed significant increases in wound closure and migration rate values when tested individually on HaCaT cells over a period of 24 h, and the wound gaps were almost completely closed at 48 h. *C. longa* extract (curcumin) did not exhibit any wound healing properties when tested individually as compared to the untreated control group. For this reason, it was not included in any combinations for further wound healing studies.

Combinations of *B. frutescens* extract with *A. vera* gel showed excellent wound healing results. Combinations 1A-1B, 1A-2B and 1A-3B (refer to section 4.3.2.1) induced the highest wound closure and migration rate ratio values. These combinations exhibited wound closure ratio values varying between 1.92 to 2.41 and 2.38 to 2.50 at 12 h and 24 h, respectively. It was evident that these combinations of *B. frutescens* extract and *A. vera* gel could potentially be advantageous for wound healing and be useful as a wound healing agent. Although *A. vera* gel and *B. frutescens* gel exhibited wound healing properties when tested individually, combinations of these plant materials showed unfavourable results and lowered the wound healing properties of these individual plant materials. In fact, combinations 1A-1C, 2A-1C, 2A-2C, 2A-3C, 3A-1C and 3A-2C (refer to section 4.3.2.1 - 4.3.2.3) demonstrated lower wound closure and migration rate ratio values than the untreated control group (i.e. values < 1). Therefore, it can be concluded that *A. vera* gel and *B. frutescens* gel are better used as individual wound healing agents, than in

combination with each other. All the *B. frutescens* aqueous extract and *B. frutescens* gel combinations demonstrated an increased tendency to enhance wound closure as compared to their individual performances. These combinations exhibited wound closure ratio values varying from 1.12 to 1.55 and 1.36 to 1.44 at 12 h and 24 h, respectively. The wounds treated with *B. frutescens* aqueous extract and *B. frutescens* gel combinations were almost completely closed at 24 h.

5.2 Future recommendations

Based on the results obtained and conclusions made, the following future recommendations are endorsed for further studies:

- Determine the *in vitro* wound healing effects of both the individual plant materials as well as the combinations of plant materials with the wound healing scratch assay in an additional skin cell model such as the fibroblast cell model. The reason for this is that fibroblasts are one of the three main cells of the skin (López-García *et al*., 2014:44) and will provide further insights into the wound healing effects of the medicinal plants investigated.
- Perform more in-depth cell culture research on the selected medicinal plant materials using more advanced 3D cell culture models. Advanced 3D cell culture models exhibit better recapitulation of *in vivo* models and can predict the efficiency of drugs more accurately.
- Identify and isolate the specific phytochemicals responsible for the wound healing characteristics of *B. frutescens* aqueous extract and *B. frutescens* gel.
- Investigate binary combinations of these plant materials and apply isobologram analysis to determine if any synergistic or antagonistic effects exist.
- Formulate the combinations of plant materials with the highest wound healing effects into different dosage forms (e.g. gels, creams, ointments, dressings or sprays) for more convenient treatment of skin wounds.
- Combine the individual plant materials or combinations of plant materials with Absorbatox (specific zeolite derivative) or metal cations (e.g. colloidal silver or zinc oxide) to see if more enhanced wound healing effects could be obtained.
- Formulate the combinations of plant materials in nanoparticles or nanofibers for better topical drug delivery, to obtain controlled drug release when applied to treat skin wounds, as well as improved bioavailability and stability.
- Determine the stability of the plant materials individually, as well as in combinations by performing stability studies.

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APPENDIX A: MTT DATA

Table A.1: MTT results for *A. vera* gel after 24 h exposure period to HaCaT cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	103,36	102,12	104,47	81,93	103,32	104,21	99,90	8,84
0.25	99,15	102,53	104,00	83,60	101,28	92,22	97,13	7,81
0.5	81,94	88,84	92,12	88,66	82,18	80,78	85,75	4,70
0.75	59,19	73,07	74,26	89,05	80,98	71,24	74,63	10,01
1	56,67	57,80	62,53	70,48	63,33	59,83	61,77	4,99
1.25	55,10	50,16	50,26	41,41	48,18	49,21	49,05	4,44
1.5	35,43	36,51	37,06	34,43	36,70	32,17	35,38	1,84
2	24,62	20,84	23,38	20,90	19,65	18,62	21,34	2,26
Dead	1,83	2,10	2,02	2,28	2,01	2,13	2,06	0,15

Table A.2: MTT results for *C. longa* extract (curcumin) after 24 h exposure period to HaCaT cells

24h Concentration (µg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
1	102,49	98,42	89,21	94,96	100,32	101,05	97,74	4,92
2.5	93,22	89,88	96,06	95,10	92,18	95,36	93,63	2,34
5	88,10	80,46	87,66	85,41	81,51	82,58	84,29	3,24
7.5	68,88	56,48	68,99	61,67	77,72	67,46	66,86	7,23
10	48,05	38,15	42,02	31,45	40,04	42,89	40,43	5,53
15	30,29	24,29	19,50	21,26	23,97	26,68	24,33	3,85
20	13,95	10,46	8,63	15,08	8,26	7,04	10,57	3,27
Dead	0,96	1,28	1,50	1,80	1,31	1,33	1,37	0,28

Table A.3: MTT results for *B. frutescens* aqueous extract after 24 h exposure period to HaCaT cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	77,41	71,82	75,22	74,40	81,72	83,31	77,31	4,44
0.25	62,70	70,79	66,94	77,54	69,89	70,72	69,76	4,90
0.5	63,46	66,03	61,24	64,42	64,13	60,92	63,37	1,97
0.75	57,58	56,27	62,92	62,29	59,95	56,18	59,20	2,98
1	58,80	56,46	63,69	63,54	62,07	61,30	60,97	2,84
1.25	59,66	60,81	69,09	70,98	62,43	58,66	63,60	5,17
1.5	55,76	56,65	55,60	67,16	53,59	57,27	57,67	4,81
2	58,68	50,51	51,03	55,72	56,89	54,24	54,51	3,25
Dead	1,56	1,87	1,79	2,02	1,31	1,29	1,64	0,30

Table A.4: MTT results for *B. frutescens* gel after 24 h exposure period to HaCaT cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	101,45	92,09	89,48	84,00	81,95	91,35	90,05	6,90
0.25	89,67	102,04	102,25	86,81	98,81	80,72	93,38	8,95
0.5	78,43	91,84	88,08	88,44	74,58	79,33	83,45	6,89
0.75	77,74	81,85	99,81	88,10	94,54	88,03	88,35	8,06
1	85,52	85,17	101,24	80,01	89,94	87,59	88,25	7,17
1.25	92,50	93,33	110,61	98,83	92,62	86,75	95,77	8,21
1.5	85,30	100,06	100,89	93,37	96,11	92,39	94,69	5,74
2	82,35	79,45	99,13	79,03	75,59	86,69	83,71	8,42
Dead	2,32	3,53	2,12	2,11	2,04	1,88	2,33	0,60

Table A.5: MTT results for *A. vera* gel after 48 h exposure period to HaCaT cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	101,73	100,70	102,06	99,32	96,20	100,12	100,02	2,13
0.25	103,99	103,85	102,83	101,22	101,03	105,14	103,01	1,64
0.5	104,26	102,59	105,09	105,79	104,75	104,80	104,55	1,08
0.75	93,21	100,15	100,49	99,72	102,56	96,33	98,74	3,38
1	69,13	75,48	80,00	82,58	82,56	77,65	77,90	5,12
1.25	30,07	29,14	26,17	30,71	29,65	29,58	29,22	1,58
1.5	4,22	4,00	3,60	5,50	3,57	3,14	4,00	0,82
2	1,93	1,59	1,27	1,19	1,38	0,94	1,38	0,34
Dead	1,86	1,53	1,86	1,83	1,64	2,28	1,83	0,26

Table A.6: MTT results for *C. longa* extract (curcumin) after 48 h exposure period to HaCaT cells

48h Concentration (µg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
1	104,82	105,05	95,37	104,46	100,74	100,86	101,88	3,74
2.5	84,58	69,01	75,67	78,31	72,99	74,30	75,81	5,29
5	61,09	59,18	56,20	62,73	63,55	65,86	61,44	3,42
7.5	31,81	17,87	21,86	31,69	19,24	23,76	24,37	6,07
10	15,25	13,10	15,01	16,66	14,20	7,65	13,64	3,16
15	3,18	3,35	3,30	4,99	3,68	2,59	3,51	0,81
20	1,94	2,00	2,95	3,42	2,72	2,05	2,51	0,61
Dead	1,37	1,79	1,43	1,56	1,57	1,39	1,52	0,16

Table A.7: MTT results for *B. frutescens* aqueous extract after 48 h exposure period to HaCaT cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	100,94	98,07	98,77	97,91	100,40	96,22	98,72	1,74
0.25	82,08	97,72	96,21	88,96	101,39	87,32	92,28	7,32
0.5	81,32	90,30	92,39	85,18	89,35	94,63	88,86	4,87
0.75	69,93	85,87	75,69	84,41	81,25	81,60	79,79	5,96
1	69,70	89,17	90,17	86,79	81,35	94,77	85,32	8,83
1.25	87,21	86,28	87,38	80,61	87,88	88,71	86,34	2,92
1.5	93,23	80,30	90,85	88,19	79,47	80,94	85,50	6,00
2	83,78	87,36	89,49	88,91	92,81	86,29	88,11	3,07
Dead	2,40	2,05	2,17	2,07	2,89	2,09	2,28	0,32

Table A.8: MTT results for *B. frutescens* gel after 48 h exposure period to HaCaT cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	102,22	103,55	95,10	103,43	94,16	83,49	96,99	7,82
0.25	93,94	96,27	95,08	94,30	92,01	78,29	91,65	6,69
0.5	98,77	99,94	98,59	101,86	97,45	91,29	97,98	3,60
0.75	94,11	98,53	100,13	97,98	92,33	95,19	96,38	2,97
1	93,09	97,55	85,36	79,45	88,72	74,01	86,37	8,68
1.25	94,75	89,58	84,07	85,76	90,49	80,23	87,48	5,17
1.5	89,24	96,80	84,02	87,32	88,15	78,49	87,34	6,05
2	80,86	101,50	91,86	90,02	83,83	78,10	87,70	8,57
Dead	1,32	1,28	1,28	2,08	1,78	1,25	1,50	0,35

Table A.9: MTT results for *A. vera* gel after 24 h exposure period to 84BR cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	97,44	99,22	99,33	114,04	93,58	97,87	100,25	7,07
0.25	92,27	86,19	83,04	87,23	94,09	72,90	85,95	7,57
0.5	80,80	80,64	89,32	88,90	88,16	79,28	84,52	4,73
0.75	69,61	69,84	57,37	65,21	71,64	80,17	68,97	7,51
1	56,25	52,80	55,99	51,29	59,40	57,57	55,55	3,01
1.25	55,43	52,82	47,06	47,94	50,83	50,04	50,69	3,11
1.5	59,55	53,94	45,96	48,37	47,83	47,80	50,57	5,16
2	40,59	39,06	31,83	32,96	35,09	28,18	34,62	4,64
Dead	14,25	15,02	14,25	18,41	18,08	21,07	16,85	2,78

Table A.10: MTT results for *C. longa* extract (curcumin) after 24 h exposure period to 84BR cells

24h Concentration (µg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
1	96,00	91,54	59,34	58,33	75,03	85,39	77,60	16,15
2.5	84,74	73,63	75,21	75,64	75,14	59,96	74,05	7,98
5	79,08	56,86	49,05	49,43	47,71	43,73	54,31	12,86
7.5	66,66	47,31	46,35	40,17	34,03	45,56	46,68	10,98
10	58,37	49,15	63,22	61,08	64,95	54,99	58,63	5,83
15	66,77	48,22	52,96	41,87	51,26	46,05	51,19	8,58
20	44,01	39,71	33,06	31,04	42,53	31,93	37,05	5,72
Dead	1,97	1,67	0,42	2,42	3,32	3,12	2,15	1,06

Table A.11: MTT results for *B. frutescens* aqueous extract after 24 h exposure period to 84BR cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	89,40	94,49	89,90	92,41	112,32	93,71	95,37	8,55
0.25	92,81	109,39	100,20	90,94	93,82	94,09	96,88	6,88
0.5	110,21	109,89	108,92	117,15	115,68	107,77	111,61	3,85
0.75	110,01	130,96	110,60	147,37	116,44	111,79	121,20	15,03
1	97,54	114,29	85,54	110,74	109,22	107,83	104,20	10,73
1.25	106,08	107,20	118,51	114,44	116,14	117,16	113,26	5,30
1.5	104,77	124,02	105,01	121,02	118,62	114,07	114,58	8,19
2	110,94	116,53	123,08	126,71	113,12	101,27	115,27	9,09
Dead	6,14	4,79	7,56	4,90	3,11	3,21	4,95	1,71

Table A.12: MTT results for *B. frutescens* gel after 24 h exposure period to 84BR cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	85,39	107,18	105,61	83,85	102,88	98,29	97,20	10,21
0.25	104,86	107,31	130,54	102,94	102,66	80,20	104,75	16,01
0.5	103,26	133,67	118,70	135,32	118,29	108,74	119,66	12,91
0.75	113,44	118,20	119,27	146,87	105,14	114,31	119,54	14,29
1	108,76	136,06	129,73	100,32	112,15	82,38	111,57	19,58
1.25	108,96	133,81	152,48	147,83	133,76	117,86	132,45	16,77
1.5	109,80	139,54	135,43	150,89	123,99	127,73	131,23	14,12
2	108,78	113,91	141,10	141,88	118,80	103,01	121,25	16,54
Dead	3,73	6,46	2,56	3,29	3,38	3,35	3,79	1,36

Table A.13: MTT results for *A. vera* gel after 48 h exposure period to 84BR cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	74,50	84,70	90,11	83,66	86,72	87,99	84,61	5,46
0.25	83,36	83,75	94,68	88,42	95,87	79,02	87,52	6,72
0.5	74,45	97,94	87,54	86,97	85,10	80,44	85,41	7,86
0.75	67,95	91,17	78,80	87,13	86,60	75,57	81,20	8,68
1	54,64	76,62	65,25	68,76	74,07	52,10	65,24	10,05
1.25	37,72	54,40	41,48	45,70	54,19	42,15	45,94	6,95
1.5	28,03	30,15	35,51	35,65	47,86	29,58	34,46	7,29
2	10,39	9,78	12,94	11,75	9,68	9,90	10,74	1,32
Dead	4,33	5,37	5,09	4,25	5,00	4,23	4,71	0,50

Table A.14: MTT results for *C. longa* extract (curcumin) after 48 h exposure period to 84BR cells

48h Concentration (µg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
1	83,78	64,77	93,59	91,25	101,47	86,00	86,81	12,46
2.5	42,85	73,38	70,41	96,28	76,95	62,09	70,32	17,60
5	29,62	33,96	39,32	39,56	36,82	27,50	34,46	5,05
7.5	23,84	19,93	27,33	22,40	27,63	20,70	23,64	3,27
10	18,15	20,12	27,11	25,75	27,46	18,54	22,85	4,38
15	20,43	18,97	21,46	21,64	16,19	8,28	17,83	5,09
20	15,93	17,18	16,68	15,25	16,11	11,43	15,43	2,07
Dead	2,14	3,04	3,48	2,96	2,56	2,63	2,80	0,46

Table A.15: MTT results for *B. frutescens* aqueous extract after 48 h exposure period to 84BR cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	85,91	73,44	98,41	100,57	94,43	93,03	90,96	9,97
0.25	89,99	79,49	90,15	92,83	81,77	92,91	87,86	5,78
0.5	86,12	95,12	90,03	86,10	76,46	93,28	87,85	6,68
0.75	70,71	76,60	100,54	76,83	94,97	94,77	85,74	12,45
1	69,90	86,75	100,28	92,49	101,11	87,03	89,59	11,47
1.25	76,76	93,11	77,82	89,24	73,70	85,86	82,75	7,76
1.5	74,11	75,16	66,67	68,94	88,14	84,07	76,18	8,41
2	66,45	73,37	69,46	88,39	74,64	75,08	74,56	7,55
Dead	2,99	2,95	3,90	3,16	2,99	2,82	3,13	0,39

Table A.16: MTT results for *B. frutescens* gel after 48 h exposure period to 84BR cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	86,50	103,99	104,22	92,18	93,84	87,45	94,70	7,79
0.25	91,43	103,93	105,69	95,40	94,36	84,31	95,85	7,97
0.5	110,43	101,94	113,67	123,57	109,50	93,03	108,69	10,40
0.75	104,49	125,17	120,05	91,30	102,00	91,03	105,67	14,30
1	100,90	103,41	112,78	96,35	103,99	91,07	101,42	7,39
1.25	92,68	95,59	103,03	108,06	110,10	95,07	100,76	7,35
1.5	88,86	102,06	113,51	111,70	100,72	91,31	101,36	10,12
2	95,03	111,27	96,33	97,10	87,14	72,39	93,21	12,84
Dead	4,54	6,21	5,60	5,04	5,67	4,68	5,29	0,65

Table A.17: MTT results for 0.5% *(v/v)* DMSO in growth media (vehicle control) after 24 h and 48 h exposure periods to HaCaT and 84BR cells

		% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
HaCaT	24 h	82,48	98,43	98,59	81,97	99,72	102,59	93,96	9,22
	48 h	99,86	104,05	103,68	101,97	99,81	104,32	102,28	2,06
84BR	24 h	106,48	107,54	132,71	115,24	138,22	136,23	122,74	14,65
	48 h	75,50	76,77	79,08	99,11	85,84	81,35	82,94	8,73

APPENDIX B: NR DATA

Table B.1: NR results for *A. vera* gel after 24 h exposure period to HaCaT cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	91,71	88,34	103,61	157,61	110,77	85,30	106,22	26,97
0.25	94,66	104,18	103,99	111,55	103,86	77,47	99,28	11,96
0.5	67,92	88,17	104,64	126,14	114,38	86,11	97,90	21,20
0.75	77,08	95,73	118,58	94,14	93,39	78,87	92,97	14,94
1	68,08	78,08	105,74	114,27	70,14	64,01	83,39	21,29
1.25	58,18	69,77	66,27	70,89	70,08	52,33	64,59	7,62
1.5	45,96	45,00	42,92	36,86	39,99	32,08	40,47	5,30
2	10,82	10,05	7,26	5,72	7,49	5,18	7,75	2,27
Dead	12,36	10,64	8,89	3,93	2,83	7,29	7,66	3,74

Table B.2: NR results for *C. longa* extract (curcumin) after 24 h exposure period to HaCaT cells

24h Concentration (µg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
1	107,65	112,12	120,38	105,47	123,66	104,25	112,25	8,09
2.5	107,85	118,17	94,63	97,64	93,97	86,23	99,75	11,42
5	62,19	67,55	62,52	83,16	68,97	46,92	65,22	11,77
7.5	69,86	63,29	47,45	49,17	60,24	59,68	58,28	8,55
10	36,41	30,82	35,15	26,09	26,36	29,17	30,66	4,36
15	30,55	36,67	36,57	26,09	30,32	34,16	32,39	4,15
20	30,55	24,37	34,98	18,98	22,39	20,77	25,34	6,18
Dead	2,66	3,84	2,08	2,02	2,68	1,49	2,46	0,81

Table B.3: NR results for *B. frutescens* aqueous extract after 24 h exposure period to HaCaT cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Ag.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	131,38	147,23	142,13	142,93	141,49	119,53	137,45	10,21
0.25	141,58	161,06	162,47	154,29	143,63	129,43	148,74	12,82
0.5	153,80	157,83	169,65	182,65	159,87	134,10	159,65	16,25
0.75	149,98	184,03	172,30	168,76	189,04	153,58	169,62	15,72
1	167,36	187,91	175,30	184,76	181,74	161,77	176,47	10,28
1.25	154,32	206,54	181,37	176,49	197,71	160,73	179,52	20,31
1.5	181,40	194,96	221,07	213,31	198,17	156,48	194,23	23,19
2	196,43	203,24	204,21	207,39	198,20	173,95	197,24	12,10
Dead	1,40	1,37	0,86	4,55	1,59	1,50	1,88	1,33

Table B 4: NR results for *B. frutescens* gel after 24 h exposure period to HaCaT cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	102,82	105,21	119,37	113,65	109,63	105,49	109,36	6,23
0.25	101,77	102,98	92,57	98,23	98,14	84,85	96,42	6,73
0.5	106,64	102,76	96,99	105,35	98,93	86,88	99,59	7,24
0.75	94,85	107,77	84,26	98,17	89,22	76,83	91,85	10,89
1	109,07	108,92	110,53	110,61	100,45	96,62	106,03	5,98
1.25	99,89	111,66	99,55	92,76	105,38	97,18	101,07	6,61
1.5	104,56	107,04	93,33	97,21	91,38	91,47	97,50	6,81
2	77,11	98,68	97,02	97,02	88,26	70,63	88,12	11,80
Dead	2,28	1,89	0,65	1,46	1,55	1,18	1,50	0,56

Table B 5: NR results for *A. vera* gel after 48 h exposure period to HaCaT cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	89,82	128,38	140,03	139,13	100,77	114,63	118,79	20,68
0.25	85,59	108,09	127,11	107,09	128,91	93,69	108,41	17,38
0.5	60,75	79,94	89,99	87,97	98,55	54,40	78,60	17,45
0.75	63,26	124,63	95,34	94,54	60,19	83,88	86,97	23,81
1	59,90	64,84	64,81	96,78	67,05	67,90	70,21	13,31
1.25	40,34	42,24	52,26	51,14	46,13	45,06	46,20	4,74
1.5	32,82	24,43	24,16	31,68	29,71	17,94	26,79	5,65
2	2,20	4,82	5,82	2,56	2,05	3,56	3,50	1,54
Dead	0,32	0,17	0,02	1,73	2,02	1,87	1,02	0,94

Table B.6: NR results for *C. longa* extract (curcumin) after 48 h exposure period to HaCaT cells

48h Concentration (µg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
1	91,65	106,15	119,11	84,89	113,06	97,30	102,03	13,07
2.5	90,54	105,72	106,02	107,81	99,94	72,26	97,05	13,69
5	24,75	23,75	25,26	24,47	23,90	27,44	24,93	1,35
7.5	32,60	22,59	35,07	24,11	24,02	24,81	27,20	5,25
10	11,78	6,98	6,94	9,50	8,82	10,84	9,15	1,98
15	1,80	1,63	2,06	2,93	3,27	2,21	2,32	0,65
20	1,76	1,74	1,99	2,95	1,63	1,38	1,91	0,55
Dead	1,87	3,45	0,92	2,75	0,28	0,68	1,66	1,25

Table B.7: NR results for *B. frutescens* aqueous extract after 48 h exposure period to HaCaT cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	104,50	110,11	112,45	113,28	98,61	112,06	108,50	5,79
0.25	122,53	132,34	120,34	115,14	137,95	128,80	126,18	8,40
0.5	133,98	150,65	153,52	132,83	111,59	120,00	133,76	16,48
0.75	132,41	174,86	149,66	166,51	145,97	134,08	150,58	17,13
1	153,15	173,05	206,44	218,15	176,30	147,39	179,08	28,27
1.25	138,12	164,69	205,45	193,28	180,18	176,11	176,31	23,43
1.5	153,53	167,76	210,34	165,42	182,75	167,48	174,55	19,85
2	166,35	180,46	221,26	175,44	166,38	163,23	178,85	21,76
Dead	7,47	1,13	11,42	2,50	0,23	3,85	4,43	4,26

Table B.8: NR results for *B. frutescens* gel after 48 h exposure period to HaCaT cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	97,93	110,05	130,38	116,68	121,35	106,75	113,86	11,45
0.25	105,35	117,72	133,80	135,35	100,99	96,65	114,98	16,74
0.5	122,33	127,28	141,20	150,76	161,57	128,42	138,59	15,35
0.75	120,21	148,94	142,74	194,48	174,54	117,27	149,70	30,31
1	123,35	142,29	168,83	183,67	156,90	123,29	149,72	24,57
1.25	119,47	143,16	155,11	163,18	171,09	101,87	142,31	26,78
1.5	112,95	152,29	148,08	177,29	167,00	111,31	144,82	27,39
2	94,48	111,73	113,25	127,10	127,99	103,74	113,05	13,07
Dead	1,35	0,51	2,20	1,85	2,15	0,69	1,46	0,73

Table B.9: NR results for *A. vera* gel after 24 h exposure period to 84BR cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	90,70	71,31	126,50	100,10	95,48	100,40	97,41	17,84
0.25	103,08	82,65	76,53	99,35	106,96	94,58	93,86	11,94
0.5	101,59	112,48	115,76	132,17	110,24	106,36	113,10	10,55
0.75	107,71	82,65	80,26	101,44	78,18	90,26	90,08	12,11
1	68,78	87,57	93,39	87,72	62,22	86,23	80,98	12,42
1.25	75,94	109,94	126,50	65,35	91,15	82,50	91,90	22,68
1.5	75,94	100,99	110,24	86,23	104,72	62,22	90,06	18,61
2	53,42	54,16	15,24	34,70	24,34	45,07	37,82	15,90
Dead	10,87	6,88	7,60	6,38	2,92	2,89	6,26	3,03

Table B.10: NR results for *C. longa* extract (curcumin) after 24 h exposure period to 84BR cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
1	97,63	73,11	62,39	79,16	73,54	64,51	75,06	12,68
2.5	52,10	108,56	89,99	93,07	64,62	50,93	76,54	23,98
5	24,08	49,55	26,73	45,09	30,55	15,69	31,95	12,95
7.5	24,08	23,44	39,47	37,13	20,57	13,78	26,41	9,93
10	46,47	43,82	57,08	50,93	49,66	38,30	47,71	6,44
15	42,54	48,70	49,44	58,04	40,42	49,44	48,10	6,21
20	51,67	61,86	66,21	48,70	57,62	43,92	55,00	8,40
Dead	1,82	1,15	1,48	2,69	2,72	2,71	2,09	0,70

Table B.11: NR results for *B. frutescens* aqueous extract after 24 h exposure period to 84BR cells

24h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	109,25	105,21	100,40	108,58	123,58	107,61	109,10	7,78
0.25	107,23	126,75	96,17	128,29	123,48	97,13	113,18	14,84
0.5	104,06	160,99	133,29	136,27	170,03	110,02	135,77	26,40
0.75	111,84	146,95	157,33	146,85	176,18	123,96	143,85	23,09
1	123,00	162,53	127,62	155,70	116,36	134,35	136,59	18,53
1.25	153,68	186,57	140,79	126,37	151,08	110,11	144,77	26,16
1.5	101,94	170,41	151,56	132,33	170,12	103,19	138,26	31,02
2	113,86	168,58	120,21	170,41	121,94	77,70	128,79	35,41
Dead	3,33	2,93	3,48	3,21	2,72	0,61	2,71	1,07

Table B.12: NR results for *B. frutescens* gel after 24 h exposure period to 84BR cells

Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	96,04	117,21	154,81	97,48	124,49	85,06	112,51	25,32
0.25	90,49	130,92	79,85	111,88	104,25	105,96	103,89	17,64
0.5	82,66	138,01	108,02	106,74	93,37	111,14	106,66	18,75
0.75	92,88	106,62	96,40	108,65	105,86	99,14	101,59	6,36
1	90,66	87,60	106,89	122,12	107,13	94,42	101,47	13,02
1.25	113,35	117,62	118,04	111,02	123,32	114,49	116,31	4,33
1.5	125,08	145,94	139,02	150,87	109,75	83,98	125,77	25,36
2	84,32	125,10	135,72	117,62	102,07	77,53	107,06	23,12
Dead	8,63	4,99	4,65	9,90	8,78	12,91	8,31	3,11

Table B.13: NR results for *A. vera* gel after 48 h exposure period to 84BR cells

48h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	98,88	104,40	124,56	111,38	98,39	78,41	102,67	15,36
0.25	87,50	108,11	109,43	122,42	107,08	69,43	100,66	18,95
0.5	74,17	106,10	111,67	120,12	112,75	76,80	100,27	19,73
0.75	98,29	104,30	111,13	120,85	98,83	60,44	98,97	20,69
1	86,23	97,31	121,05	121,83	102,78	75,78	100,83	18,48
1.25	75,24	98,73	83,15	98,24	82,22	57,90	82,58	15,29
1.5	64,30	77,93	97,66	95,31	71,48	63,91	78,43	14,92
2	43,35	55,12	51,51	73,68	46,62	40,81	51,85	11,91
Dead	1,92	2,97	2,11	3,61	1,69	2,65	2,49	0,72

Table B.14: NR results for *C. longa* extract (curcumin) after 48 h exposure period to 84BR cells

48h Concentration (µg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
1	96,97	119,40	113,12	113,89	129,08	81,06	108,92	17,19
2.5	75,08	85,55	86,80	97,93	96,25	57,68	83,22	14,99
5	59,65	80,17	68,50	94,70	77,29	48,17	71,41	16,37
7.5	33,94	30,47	37,83	25,15	28,97	27,48	30,64	4,59
10	33,70	34,60	29,33	31,19	30,77	29,45	31,51	2,19
15	29,93	39,80	37,17	45,66	50,56	33,40	39,42	7,68
20	13,25	23,05	21,98	16,12	13,43	13,43	16,87	4,51
Dead	3,04	0,38	1,99	1,50	1,68	1,71	1,72	0,85

Table B.15: NR results for *B. frutescens* aqueous extract after 48 h exposure period to 84BR cells

48 h Concentration (mg/ml)	% Cell viability Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	70,10	87,08	106,92	99,02	111,50	76,11	91,79	16,78
0.25	83,50	100,27	96,67	96,67	84,48	79,63	90,20	8,65
0.5	83,65	97,47	97,32	100,36	129,82	76,05	97,45	18,47
0.75	101,91	120,38	116,42	124,49	123,99	91,31	113,08	13,51
1	113,20	120,23	118,12	121,16	98,22	83,74	109,11	15,03
1.25	115,44	129,05	119,13	109,30	107,24	120,08	116,71	7,95
1.5	109,57	117,58	128,63	113,11	116,93	123,09	118,15	6,86
2	111,21	102,86	125,15	101,32	110,01	100,06	108,44	9,39
Dead	3,13	1,52	0,92	2,32	0,98	0,00	1,48	1,11

Table B.16: NR results for *B. frutescens* gel after 48 h exposure period to 84BR cells

48 h Concentration (mg/ml)	% Cell vaibility Replicates 1	2	3	4	5	6	Avg.	Stdev
Untreated control	100	100	100	100	100	100	100	0
0.1	139,10	117,05	131,59	111,66	117,55	107,01	120,66	12,24
0.25	95,39	142,21	146,94	129,25	113,31	118,35	124,24	19,23
0.5	104,28	149,58	148,35	137,67	142,43	94,21	129,42	23,97
0.75	90,96	152,45	161,86	149,97	142,95	125,06	137,21	25,78
1	106,24	129,11	110,09	129,33	127,62	95,34	116,29	14,43
1.25	75,06	127,04	126,85	102,41	104,17	94,76	105,05	19,86
1.5	107,42	163,73	135,74	116,34	123,66	73,51	120,07	30,02
2	102,52	109,54	114,80	125,17	94,24	84,28	105,09	14,66
Dead	2,59	0,03	1,61	3,19	1,79	0,11	1,18	1,56

Table B.17: NR results for 0.5% *(v/v)* DMSO in growth media (vehicle control) after 24 h and 48 h exposure periods to HaCaT and 84BR cells

		% Cell viability Replicates							
		1	2	3	4	5	6	Avg.	Stdev
HaCaT	24 h	89,50	106,98	121,61	120,40	109,18	80,81	104,75	16,49
	48 h	81,98	96,28	95,31	95,49	112,55	81,60	93,87	11,41
84BR	24 h	110,41	118,06	127,10	103,26	111,60	78,83	108,21	16,48
	48 h	76,15	111,46	100,82	113,65	107,24	75,48	97,47	17,33

APPENDIX C: WOUND CLOSURE DATA FOR INDIVIDUAL PLANT MATERIALS

Table C.1: Wound closure data for *A. vera* gel after 24 h exposure period to HaCaT cells

24h	% Wound closure ratios Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,686	1,152	1,259	1,366	0,282
0.5	1,261	0,869	1,158	1,096	0,203
0.75	1,183	1,278	1,307	1,256	0,065

Table C.2: Wound closure data for *A. vera* gel after 48 h exposure period to HaCaT cells

48h	% Wound closure ratios Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,484	1,173	1,603	1,420	0,222
0.5	1,413	1,106	1,295	1,271	0,155
0.75	1,175	1,262	1,334	1,257	0,080

Table C.3: Wound closure data for *C. longa* extract (curcumin) after 24 h exposure period to HaCaT cells

24h	% Wound closure ratio Replicates				
Concentration (µg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
1	0,766	1,322	0,779	0,956	0,317
2.5	0,797	0,463	0,506	0,589	0,182
5	0,262	0,387	0,195	0,281	0,097

Table C.4: Wound closure data for *C. longa* extract (curcumin) after 48 h exposure period to HaCaT cells

48h	% Wound closure ratio Replicates				
Concentration (µg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
1	0,906	1,110	0,810	0,942	0,153
2.5	0,728	0,839	0,841	0,803	0,065
5	0,468	0,577	0,454	0,500	0,068

Table C.5: Wound closure data for *B. frutescens* aqueous extract after 24 h exposure period to HaCaT cells

24h	% Wound closure ratio Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,604	1,275	1,631	1,503	0,198
0.5	1,607	1,675	1,645	1,642	0,034
0.75	1,687	1,714	1,708	1,703	0,014

Table C.6: Wound closure data for *B. frutescens* aqueous extract after 48 h exposure period to HaCaT cells

48h	% Wound closure ratio Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,027	1,027	1,027	1,027	0
0.5	1,027	1,027	1,027	1,027	0
0.75	1,027	1,027	1,027	1,027	0

Table C.7: Wound closure data for *B. frutescens* gel after 24 h exposure period to HaCaT cells

24h	% Wound closure ratio Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,501	1,952	1,837	1,763	0,234
0.5	1,607	1,826	2,092	1,842	0,243
0.75	1,510	1,120	1,509	1,380	0,225

Table C.8: Wound closure data for *B. frutescens* gel after 48 h exposure period to HaCaT cells

	% Wound closure ratio				
48h	Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,238	1,307	1,307	1,284	0,040
0.5	1,251	1,300	1,307	1,286	0,030
0.75	1,307	1,245	1,307	1,286	0,036

APPENDIX D: MIGRATION RATE DATA FOR INDIVIDUAL PLANT MATERIALS

Table D.1: Migration rate data for *A. vera* gel after 24 h exposure period to HaCaT cells

24h	Migration rate ($\mu m^2/h$) ratios Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,5861	1,0549	1,3467	1,3292	0,2661
0.5	1,3105	0,8596	0,9955	1,0552	0,2313
0.75	1,2385	1,3350	1,2062	1,2599	0,0670

Table D.2: Migration rate data for *A. vera* gel after 48 h exposure period to HaCaT cells

48h	Migration rate ($\mu m^2/h$) ratios Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,4003	1,0773	1,7201	1,3992	0,3214
0.5	1,4735	1,0979	1,1174	1,2296	0,2114
0.75	1,2347	1,3234	1,2357	1,2646	0,0509

Table D.3: Migration rate data for *C. longa* extract (curcumin) after 24 h exposure period to HaCaT cells

24h	Migration rate ($\mu m^2/h$) ratio Replicates				
Concentration (µg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
1	0,612	0,956	0,727	0,765	0,175
2.5	0,817	0,407	0,476	0,567	0,220
5	0,240	0,377	0,181	0,266	0,100

Table D.4: Migration rate data for *C. longa* extract (curcumin) after 48 h exposure period to HaCaT cells

24h	Migration rate (μm²/h) ratio Replicates				
Concentration (μg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
1	0,723	0,803	0,755	0,760	0,134
2.5	0,745	0,737	0,790	0,758	0,031
5	0,428	0,562	0,421	0,470	0,166

Table D.5: Migration rate data for *B. frutescens* aqueous extract after 24 h exposure period to HaCaT cells

24h	Migration rate (μm²/h) ratio Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,452	1,180	1,503	1,379	0,174
0.5	2,011	1,613	1,368	1,664	0,324
0.75	1,616	1,680	1,633	1,643	0,033

Table D.6: Migration rate data for *B. frutescens* aqueous extract after 48 h exposure period to HaCaT cells

48h	Migration rate (μm²/h) ratio Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	0,930	0,951	0,947	0,943	0,011
0.5	1,285	0,990	0,854	1,043	0,220
0.75	0,984	1,007	0,982	0,991	0,014

Table D.7: Migration rate data for *B. frutescens* gel after 24 h exposure period to HaCaT cells

24h	Migration rate (μm²/h) ratio Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,534	1,698	1,534	1,589	0,095
0.5	1,417	1,436	1,539	1,464	0,066
0.75	1,117	0,918	1,264	1,100	0,174

Table D.8: Migration rate data for *B. frutescens* gel after 48 h exposure period to HaCaT cells

48h	Migration rate ($\mu m^2/h$) ratio Replicates				
Concentration (mg/ml)	1	2	3	Average	Stdev
Untreated control	1	1	1	1	0
0.25	1,265	1,137	1,091	1,164	0,090
0.5	1,102	1,022	0,961	1,028	0,071
0.75	0,967	1,020	1,094	1,027	0,064

APPENDIX E: WOUND CLOSURE DATA FOR COMBINATION PLANT MATERIALS

Table E.1: Wound closure data for combinations 1A-1B, 1A-2B and 1A-3B after 12 h exposure period to HaCaT cells

	% Wound closure ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1A-1B	2,444	1,703	2,087	1,533	1,651	2,088	1,917	0,346
1A-2B	2,502	2,404	2,645	1,912	1,398	1,448	2,051	0,546
1A-3B	1,671	2,516	1,668	2,996	2,658	2,925	2,406	0,596

Table E.2: Wound closure data for combinations 1A-1B, 1A-2B and 1A-3B after 24 h exposure period to HaCaT cells

	% Wound closure ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1A-1B	2,800	2,271	2,382	1,933	2,301	2,569	2,376	0,293
1A-2B	2,762	2,654	2,800	2,470	2,100	2,083	2,478	0,321
1A-3B	2,143	2,506	2,008	2,778	2,757	2,800	2,499	0,347

Table E.3: Wound closure data for combinations 1A-1C, 1A-2C and 1A-3C after 12 h exposure period to HaCaT cells

	% Wound closure ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1A-1C	0,519	1,067	0,664	1,020	0,238	1,158	0,778	0,363
1A-2C	0,703	1,314	0,610	1,983	0,872	1,769	1,209	0,575
1A-3C	1,620	1,310	0,717	1,157	0,603	0,622	1,005	0,421

Table E.4: Wound closure data for combinations 1A-1C, 1A-2C and 1A-3C after 24 h exposure period to HaCaT cells

	% Wound closure ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1A-1C	0,492	0,955	0,678	0,990	0,348	1,142	1,209	0,575
1A-2C	0,799	1,439	0,632	1,619	0,840	1,596	1,154	0,445
1A-3C	1,321	1,104	0,735	1,065	0,975	0,782	0,997	0,218

Table E.5: Wound closure data for combinations 2A-1B, 2A-2B and 2A-3B after 12 h exposure period to HaCaT cells

	% Wound closure ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2A-1B	1,157	0,958	1,180	1,295	1,153	1,396	1,190	0,148
2A-2B	1,021	0,976	1,126	1,068	1,067	1,163	1,070	0,068
2A-3B	1,249	1,470	1,299	1,332	1,693	1,444	1,414	0,161

Table E.6: Wound closure data for combinations 2A-1B, 2A-2B and 2A-3B after 24 h exposure period to HaCaT cells

	% Wound closure ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2A-1B	1,340	1,373	1,265	1,404	1,258	1,413	1,342	0,068
2A-2B	1,027	1,085	1,192	1,140	1,089	1,139	1,112	0,058
2A-3B	1,341	1,390	1,269	1,344	1,251	1,395	1,332	0,060

Table E.7: Wound closure data for combinations 2A-1C, 2A-2C and 2A-3C after 12 h exposure period to HaCaT cells

12h	% Wound closure ratios Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2A-1C	0,339	0,287	0,327	0,647	0,373	0,376	0,392	0,129
2A-2C	0,668	0,840	0,212	0,479	1,074	0,532	0,634	0,300
2A-3C	0,629	0,260	0,250	0,467	0,582	0,584	0,462	0,169

Table E.8: Wound closure data for combinations 2A-1C, 2A-2C and 2A-3C after 24 h exposure period to HaCaT cells

24h	% Wound closure ratios Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2A-1C	0,551	0,379	0,392	0,736	0,454	0,577	0,515	0,135
2A-2C	0,368	0,843	0,338	0,509	0,736	0,473	0,545	0,203
2A-3C	0,674	0,393	0,359	0,511	0,621	0,722	0,547	0,150

Table E.9: Wound closure data for combinations 3A-1B, 3A-2B and 3A-3B after 12 h exposure period to HaCaT cells

12h	% Wound closure ratios Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3A-1B	1,157	1,255	1,035	1,134	1,116	1,238	1,156	0,082
3A-2B	1,278	1,423	1,353	1,471	1,001	1,156	1,280	0,176
3A-3B	1,291	1,135	1,532	1,101	1,427	1,028	1,253	0,198

Table E.10: Wound closure data for combinations 3A-1B, 3A-2B and 3A-3B after 24 h exposure period to HaCaT cells

	% Wound closure ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3A-1B	1,157	1,280	1,205	1,214	1,260	1,350	1,244	0,068
3A-2B	1,336	1,392	1,392	1,392	1,259	1,359	1,355	0,052
3A-3B	1,387	1,383	1,392	1,371	1,392	1,340	1,378	0,020

Table E.11: Wound closure data for combinations 3A-1C, 3A-2C and 3A-3C after 12 h exposure period to HaCaT cells

	% Wound closure ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3A-1C	0,804	0,856	0,669	0,954	0,937	0,661	0,813	0,127
3A-2C	0,552	0,950	0,524	0,599	1,023	0,971	0,770	0,234
3A-3C	0,924	1,096	0,756	0,992	1,047	1,054	0,978	0,124

Table E.12: Wound closure data for combinations 3A-1C, 3A-2C and 3A-3C after 24 h exposure period to HaCaT cells

	% Wound closure ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3A-1C	0,786	0,971	0,788	0,994	0,902	0,768	0,868	0,101
3A-2C	0,704	0,907	0,572	0,633	0,935	0,937	0,781	0,165
3A-3C	0,865	1,044	0,824	0,942	1,049	0,995	0,953	0,093

Table E.13: Wound closure data for combinations 1B-1C, 1B-2C and 1B-3C after 12 h exposure period to HaCaT cells

	% Wound closure ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1B-1C	1,260	1,400	1,350	1,315	1,565	1,485	1,396	0,113
1B-2C	1,574	1,355	1,406	1,078	1,184	1,205	1,300	0,179
1B-3C	1,555	1,475	1,539	1,515	1,730	1,465	1,547	0,096

Table E.14: Wound closure data for combinations 1B-1C, 1B-2C and 1B-3C after 24 h exposure period to HaCaT cells

	% Wound closure ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1B-1C	1,363	1,363	1,363	1,363	1,363	1,363	1,363	0
1B-2C	1,363	1,363	1,363	1,363	1,362	1,363	1,362	0
1B-3C	1,363	1,363	1,363	1,363	1,363	1,363	1,363	0

Table E.15: Wound closure data for combinations 2B-1C, 2B-2C and 2B-3C after 12 h exposure period to HaCaT cells

	% Wound closure ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2B-1C	1,360	1,314	1,232	1,350	1,745	1,411	1,402	0,178
2B-2C	1,636	1,263	1,212	1,409	1,091	1,140	1,292	0,201
2B-3C	1,410	1,045	1,073	1,030	0,943	1,230	1,122	0,170

Table E.16: Wound closure data for combinations 2B-1C, 2B-2C and 2B-3C after 24 h exposure period to HaCaT cells

	% Wound closure ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2B-1C	1,377	1,377	1,377	1,377	1,377	1,377	1,377	0
2B-2C	1,377	1,377	1,377	1,377	1,377	1,377	1,377	0
2B-3C	1,377	1,377	1,377	1,377	1,377	1,377	1,377	0

Table E.17: Wound closure data for combinations 3B-1C, 3B-2C and 3B-3C after 12 h exposure period to HaCaT cells

	% Wound closure ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3B-1C	1,237	1,324	1,210	1,114	1,254	1,341	1,247	0,082
3B-2C	1,210	1,266	0,934	1,254	1,200	1,122	1,164	0,124
3B-3C	1,439	1,274	1,230	1,139	1,059	1,135	1,213	0,135

Table E.18: Wound closure data for combinations 3B-1C, 3B-2C and 3B-3C after 24 h exposure period to HaCaT cells

	% Wound closure ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3B-1C	1,442	1,429	1,442	1,442	1,442	1,442	1,440	0,005
3B-2C	1,442	1,442	1,442	1,442	1,442	1,442	1,442	0
3B-3C	1,442	1,442	1,442	1,442	1,442	1,442	1,442	0

APPENDIX F: MIGRATION RATE DATA FOR COMBINATION PLANT MATERIALS

Table F.1: Migration rate data for combinations 1A-1B, 1A-2B and 1A-3B after 12 h exposure period to HaCaT cells

	Migration rate (µm²/h) ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1A-1B	0,733	0,773	1,130	1,521	1,468	1,894	1,253	0,457
1A-2B	2,378	2,366	2,308	1,779	1,214	1,423	1,911	0,515
1A-3B	1,823	2,278	1,804	2,749	2,447	2,967	2,345	0,475

Table F.2: Migration rate data for combinations 1A-1B, 1A-2B and 1A-3B after 24 h exposure period to HaCaT cells

	Migration rate (µm²/h) ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1A-1B	1,667	2,046	2,561	1,904	2,031	2,313	2,087	0,313
1A-2B	2,606	2,593	2,425	2,281	1,811	2,032	2,291	0,318
1A-3B	2,321	2,252	2,156	2,641	2,519	2,819	2,451	0,253

Table F.3: Migration rate data for combinations 1A-1C, 1A-2C and 1A-3C after 12 h exposure period to HaCaT cells

	Migration rate (µm²/h) ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1A-1C	0,620	1,326	0,710	1,049	0,283	1,023	0,835	0,371
1A-2C	0,629	1,192	0,627	1,862	0,846	1,634	1,132	0,525
1A-3C	1,683	1,569	0,798	1,142	0,643	0,652	1,081	0,460

Table F.4: Migration rate data for combinations 1A-1C, 1A-2C and 1A-3C after 24 h exposure period to HaCaT cells

	Migration rate ($\mu m^2/h$) ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1A-1C	0,590	1,191	0,727	1,022	0,415	1,013	0,826	0,297
1A-2C	0,718	1,310	0,652	1,527	0,818	1,481	1,084	0,399
1A-3C	1,378	1,327	0,821	1,055	1,043	0,823	1,074	0,239

Table F.5: Migration rate data for combinations 2A-1B, 2A-2B and 2A-3B after 12 h exposure period to HaCaT cells

	Migration rate ($\mu m^2/h$) ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2A-1B	0,626	0,481	0,853	0,672	0,899	0,819	0,725	0,160
2A-2B	1,134	1,083	1,008	1,018	1,054	1,218	1,086	0,079
2A-3B	1,017	0,967	1,287	1,108	1,113	1,091	1,097	0,110

Table F.6: Migration rate data for combinations 2A-1B, 2A-2B and 2A-3B after 24 h exposure period to HaCaT cells

	Migration rate ($\mu m^2/h$) ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2A-1B	0,713	0,678	0,900	0,716	0,964	0,816	0,798	0,115
2A-2B	1,122	1,183	1,050	1,069	1,057	1,172	1,109	0,059
2A-3B	1,073	0,899	1,237	1,099	0,809	1,036	1,026	0,152

Table F.7: Migration rate data for combinations 2A-1C, 2A-2C and 2A-3C after 12 h exposure period to HaCaT cells

	Migration rate ($\mu m^2/h$) ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2A-1C	0,406	0,402	0,476	0,757	0,506	0,318	0,477	0,152
2A-2C	0,891	0,937	0,209	0,622	1,307	0,724	0,782	0,366
2A-3C	0,884	0,307	0,359	0,683	0,934	0,826	0,666	0,272

Table F.8: Migration rate data for combinations 2A-1C, 2A-2C and 2A-3C after 24 h exposure period to HaCaT cells

	Migration rate ($\mu m^2/h$) ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2A-1C	0,660	0,532	0,570	0,863	0,616	0,488	0,621	0,133
2A-2C	0,491	0,942	0,333	0,663	0,896	0,644	0,662	0,233
2A-3C	0,950	0,464	0,516	0,748	0,997	1,022	0,783	0,247

Table F.9: Migration rate data for combinations 3A-1B, 3A-2B and 3A-3B after 12 h exposure period to HaCaT cells

	Migration rate ($\mu m^2/h$) ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3A-1B	1,294	1,263	1,025	1,296	1,204	1,204	1,214	0,102
3A-2B	1,370	1,429	1,370	1,424	0,998	1,216	1,301	0,167
3A-3B	1,178	1,189	1,688	1,133	1,514	1,025	1,288	0,255

Table F.10: Migration rate data for combinations 3A-1B, 3A-2B and 3A-3B after 24 h exposure period to HaCaT cells

	Migration rate ($\mu m^2/h$) ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3A-1B	1,295	1,288	1,193	1,387	1,360	1,314	1,306	0,067
3A-2B	1,433	1,399	1,410	1,348	1,255	1,431	1,379	0,068
3A-3B	1,266	1,450	1,535	1,411	1,477	1,335	1,412	0,098

Table F.11: Migration rate data for combinations 3A-1C, 3A-2C and 3A-3C after 12 h exposure period to HaCaT cells

	Migration rate ($\mu m^2/h$) ratios							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3A-1C	0,812	0,801	0,626	0,820	1,008	0,545	0,769	0,163
3A-2C	0,725	0,883	0,544	0,693	1,115	0,823	0,797	0,195
3A-3C	1,056	1,310	0,653	1,274	1,173	1,167	1,105	0,239

Table F.12: Migration rate data for combinations 3A-1C, 3A-2C and 3A-3C after 24 h exposure period to HaCaT cells

	Migration rate ($\mu m^2/h$) ratios							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3A-1C	0,794	0,909	0,739	0,856	0,972	0,635	0,817	0,121
3A-2C	0,926	0,844	0,594	0,733	1,021	0,796	0,819	0,130
3A-3C	0,990	1,249	0,713	1,212	1,177	1,103	1,074	0,214

Table F.13: Migration rate data for combinations 1B-1C, 1B-2C and 1B-3C after 12 h exposure period to HaCaT cells

12h	Migration rate ($\mu m^2/h$) ratio Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1B-1C	0,897	1,288	1,188	1,155	1,439	1,380	1,225	0,194
1B-2C	1,377	1,306	1,273	0,844	1,517	1,060	1,230	0,241
1B-3C	1,526	1,499	1,634	1,250	1,525	1,372	1,468	0,135

Table F.14: Migration rate data for combinations 1B-1C, 1B-2C and 1B-3C after 24 h exposure period to HaCaT cells

24h	Migration rate ($\mu m^2/h$) ratio Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
1B-1C	0,955	1,234	1,179	1,178	1,233	1,246	1,171	0,110
1B-2C	1,173	1,292	1,214	1,050	1,717	1,179	1,271	0,232
1B-3C	1,315	1,363	1,423	1,106	1,182	1,256	1,274	0,117

Table F.15: Migration rate data for combinations 2B-1C, 2B-2C and 2B-3C after 12 h exposure period to HaCaT cells

12h	Migration rate ($\mu m^2/h$) ratio Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2B-1C	1,625	1,454	1,207	2,096	1,287	1,625	1,549	0,318
2B-2C	1,796	1,383	1,262	1,438	1,001	1,052	1,322	0,290
2B-3C	1,543	1,097	1,248	1,125	1,013	1,423	1,242	0,205

Table F.16: Migration rate data for combinations 2B-1C, 2B-2C and 2B-3C after 24 h exposure period to HaCaT cells

	Migration rate (μm²/h) ratio							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
2B-1C	1,637	1,516	1,342	2,128	1,010	1,579	1,535	0,368
2B-2C	1,505	1,500	1,427	1,399	1,257	1,265	1,392	0,110
2B-3C	1,499	1,439	1,594	1,497	1,472	1,585	1,514	0,062

Table F.17: Migration rate data for combinations 3B-1C, 3B-2C and 3B-3C after 12 h exposure period to HaCaT cells

	Migration rate (μm²/h) ratio							
12h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3B-1C	1,310	1,975	1,173	1,006	1,272	1,644	1,397	0,352
3B-2C	1,238	1,453	1,004	1,376	1,552	1,376	1,333	0,191
3B-3C	1,453	1,468	1,417	1,355	1,116	1,405	1,369	0,130

Table F.18: Migration rate data for combinations 3B-1C, 3B-2C and 3B-3C after 24 h exposure period to HaCaT cells

	Migration rate (μm²/h) ratio							
24h	Replicates							
Concentration (mg/ml)	1	2	3	4	5	6	Average	Stdev
Untreated control	1	1	1	1	1	1	1	0
3B-1C	1,520	2,121	1,391	1,296	1,456	1,759	1,590	0,303
3B-2C	1,467	1,647	1,543	1,575	1,855	1,758	1,641	0,144
3B-3C	1,448	1,653	1,653	1,707	1,513	1,777	1,625	0,122

APPENDIX G: ETHICS APPROVAL LETTER

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North-West University Health Research Ethics Committee (NWU-HREC)

Tel: 018 299-1206
Email: Ethics-HRECApply@nwu.ac.za (for human studies)

28 February 2020

RESEARCH ETHICS COMMITTEE LETTER OF DECISION: NO RISK

Based on the review by the North-West University Health Research Ethics Committee (NWU-HREC) on 28/02/2020, the NWU-HREC hereby clears your study as a no risk study. This implies that the NWU-HREC grants its permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Combining selected medicinal plant extracts to improve wound healing of the skin
Principal Investigator/Study Supervisor/Researcher: Prof JH Hamman
Student: A Hattingh - 25878956

Ethics number:

N	W	U	-	0	0	3	4	4	-	2	0	-	A	1
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: **28/02/2020**

Risk: **No Risk**

General conditions:

The following general terms and conditions will apply:

- *The commencement date indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-HREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research;*
  - *withdraw or postpone clearance if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-HREC or that information has been false or misrepresented;*
    - *submission of the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-HREC can be contacted for further information via Ethics-HRECApply@nwu.ac.za or 018 299 1206*

The NWU-HREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-HREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by Prof Petra Bester
Date: 2020.03.03 08:33:48 +02'00'

NWU-HREC Chairperson

Digitally signed by Wayne Towers
Date: 2020.03.02 13:06:00 +02'00'

Head of the Faculty of Health Sciences Ethics Office for Research, Training and Support

Current details: (13210572) G:\My Drive\My Documents 20190227\NWU-HREC\NWU-HREC_Applications\NWU-HREC_Applications-2020\NWU-HREC_App02-20200311\NWU-00344-20-S1(JH Hamman-A Hattingh)-NR\NWU-00344-20-S1(JH Hamman-A Hattingh)-LoD\9.1.5.4.3_LOD_NWU-00344-20-A1_20200228.docm
27 February 2020

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