# Effects of selected plant materials on *in vitro* wound healing using cell culture model


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Dissertation submitted in fulfilment of the requirements for the degree *Master of Science in Pharmaceutics* at the North West University


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Graduation: May 2019

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***If-***

*by Rudyard Kipling*

*If you can keep your head when all about you*
*Are losing theirs and blaming it on you,*
*If you can trust yourself when all men doubt you,*
*But make allowance for their doubting too;*
*If you can wait and not be tired by waiting,*
*Or being lied about, don’t deal in lies,*
*Or being hated, don’t give way to hating,*
*And yet don’t look too good, nor talk too wise:*

*If you can dream—and not make dreams your master;*
*If you can think—and not make thoughts your aim;*
*If you can meet with Triumph and Disaster*
*And treat those two impostors just the same;*
*If you can bear to hear the truth you’ve spoken*
*Twisted by knaves to make a trap for fools,*
*Or watch the things you gave your life to, broken,*
*And stoop and build ’em up with worn-out tools:*

*If you can make one heap of all your winnings*
*And risk it on one turn of pitch-and-toss,*
*And lose, and start again at your beginnings*
*And never breathe a word about your loss;*
*If you can force your heart and nerve and sinew*
*To serve your turn long after they are gone,*
*And so hold on when there is nothing in you*
*Except the Will which says to them: ‘Hold on!’*

*If you can talk with crowds and keep your virtue,*
*Or walk with Kings—nor lose the common touch,*
*If neither foes nor loving friends can hurt you,*
*If all men count with you, but none too much;*
*If you can fill the unforgiving minute*
*With sixty seconds’ worth of distance run,*
*Yours is the Earth and everything that’s in it,*
*And—which is more—you’ll be a Man, my son!*

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# ACKNOWLEDGEMENTS

**Praise and thanks to the triune God**, who guides my feet and lights my path. Fearing Him is truly the beginning of knowledge and understanding.

I would like to thank my supervisor, **Prof. Jan Steenekamp** for your guidance and support. You set a great example for what a supervisor should be. I would also like thank you for being willing to take on a project that was somewhat out of the ordinary for you. It was a great privilege to have you as my supervisor.

Thanks to **Prof. Sias Hamman**, my co-supervisor. It was truly privilege learning from your vast experience in research and especially in writing. Without your extremely thoughtful critique and comment, this project would not have been successful.

Thanks to **Dr Clarissa Willers**, my assistant-supervisor, for taking me under your wing in the cell culture lab and for being endlessly patient with me as I learned how to become somewhat proficient in cell culture techniques. Thanks also for assisting in processing what seemed like mountains of experimental data.

I would also like to extent my gratitude to **Dr Christiaan Malherbe** for providing the honeybush extracts and characterising them. Thanks also for providing critique and comment on my research proposal.

Thanks also to **Mr Hannes and Jaap Viljoen of Rooiklip nursery** in Swellendam for providing the *Aloe muthi-muthi* leaves that were used in this research.

I would like to thank the **NWU** for providing me with a bursary. The financial support of the NWU is hereby acknowledged.

Thanks to my **parents and brother** for your endless love and support. I love all of you so much.

Last, but truly my number one - thank you, **Belinda**. I could not have asked for a better teammate to spend the rest of my life with. I love you immensely.

## ABSTRACT

Since ancient times, wounds have been treated using plant based remedies. Of these remedies, *Aloe* species especially *A. vera* is probably the most prominent. *Aloe* vera is also the most studied of the *Aloe* genus in terms of wound healing efficacy. *A. ferox* is another aloe with known use as a wound healing remedy and with wound healing effects supported in literature. Recently, a hybrid of *A. vera* and *A. ferox,* called *A. muthi-muthi,* has been cultivated. Plants rich in bioactive phytochemicals are often consumed for their perceived health benefits. Herbal infusions, or "teas", are often made from plants like *Aspalathus linearis* (rooibos) and *Cyclopia* spp. (honeybush). Although honeybush has been demonstrated to have health effects such as anti-tumour activity, the wound healing potential of this genus is mostly unknown.

In order to investigate the *in vitro* wound healing effects of *A. muthi-muthi* gel and whole leaf material, as well as *Cyclopia genistoides* extracts (both crude extracts and fractions rich in benzophenones and xanthones respectively), an appropriate model needed to be selected. Human immortalised keratinocytes (HaCaT cells) serve as an analogue to rapidly proliferating human epidermis and a scratch assay using this cell line was used to simulate wound healing *in vitro.* Induced scratches served as simulated "wounds". Wound closure was measured 24 h and 48 h after scratches into monolayers of HaCaT cells were induced. The closure rate was also subsequently determined. To determine whether the plant materials selected for this study exhibited cytotoxic effects, an methyl thiazolyl tetrazolium (MTT)-assay was performed prior to the scratch assays to determine cell viability after an exposure period of 48 h.

From the results of the MTT-assays, no severe cytotoxic effects were observed in HaCaT cells exposed to all plant materials at all experimental concentrations. None of the *Cyclopia genistoides* extracts tested displayed any improvement in wound closure or closure rate. On the other hand, a statistically significant ($p < 0.05$) improvement in percentage wound closure and closure rate was observed in HaCaT cells treated with *A. muthi-muthi* gel, corresponding with what has been found in literature with *A. vera* and *A. ferox*. Contrary to the findings of the *A. muthi-muthi* gel, no improvement in wound closure and closure rate shown in HaCaT cells treated with *A. muthi-muthi* whole leaf was found compared to an untreated control. Subsequently, a migration assay was also performed on *A. muthi-muthi* gel using the CytoSelect™ 24-well migration assay kit. No improvement in HaCaT cell migration compared to an untreated control was observed, however.

**Keywords:** Wound healing, *Aloe vera, Aloe ferox, Aloe muthi-muthi, Cyclopia*, honeybush, scratch assay

# UITTREKSEL

Sedert antieke tye is wonde behandel met medikamente wat meestal vanaf plantaardige bronne verkry is. Aalwynspesies, veral *Aloe vera* is waarskynlik die bekendste voorbeeld van hierdie plante. Wat wondgenesingsdoeltreffendheid betref is *A. vera* die mees nagevorsde spesie in die *Aloe* genus. Nog 'n aalwyn met 'n gevestigde gebruik as wondgenesingsraat is *A. ferox*, met wondgenesingseffekte wat in die literatuur opgeteken is. Daar is onlangs 'n hibried van *A. vera* en *A. ferox* gekweek, naamlik *A. muthi-muthi*. Plante wat ryk is in bioaktiewe en fitochemiese stowwe word geredelik gebruik vir hul waargenome gesondheidsvoordele. Infusies of "tees" word berei van plante soos *Aspalathus linearis* (rooibos) en *Cyclopia* (heuningbos) spesies. Al het heuningbos bewese gesondheidseffekte, soos anti-kankergewasaktiwiteit, is die wondgenesingspotensiaal hoofsaaklik onbekend.

Om die *in vitro* wondgenesingseffekte van *A. muthi-muthi* jel en heelblaar plantmateriaal, asook dié van *Cyclopia genistoides* ekstrakte (beide ru-ekstrakte en fraksies onderskeidelik ryk aan bensofenone en xantone) te ondersoek, moes 'n gepaste model gekies word. Menslike verontsterflike keratienosiete (HaCaT-selle) dien as 'n analoog van vinnig prolifererende menslike epidermis en 'n krapwondtoets wat gebruik maak van hierdie sellyn is gebruik om wondgenesing *in vitro* te simuleer. Geïnduseerde krappe dien hier as gesimuleerde 'wonde". Wondtoegroei is na 24 h en 48 h gemeet nadat krappe in enkellae van die HaCaT-selle geïnduseer is. Die tempo van toegroei is ook bepaal. Om vas te stel of die plantmateriaal wat vir hierdie studie uitgekies is enige sitotoksiese effekte toon, is 'n metiel tiazoliel tetrazolium (MTT)-toets voor die krapwondtoetse uitgevoer om sellewensvatbaarheid na 48 h vas te stel.

Die MTT-toetsresultate het getoon dat geen duidende sitotoksiese effekte op HaCaT selle by enige van die plantmateriaal en by enige van die toetskonsentrasies veroorsaak is nie. Geeneen van die *Cyclopia genistoides* ekstrakte wat getoets is, het enige verbetering in wond toegroei of toegroeitempo getoon nie. 'n Statistiesbetekenisvolle verbetering ($p < 0.05$) in toegroei en toegroeitempo is waargeneem in HaCaT selle behandel met *A. muthi-muthi* jel. Dit korrespondeer met bevindinge in die literatuur oor *A. vera* en *A. ferox*. In teenstelling met wat bevind is met *A. muthi-muthi* jel, is geen verbetering in toegroei of toegroeitempo waargeneem in HaCaT-selle wat behandel is met *A. muthi-muthi* heelblaar in vergelyking met 'n onbehandelde kontrolegroep nie. 'n Migrasiestudie waarin die CytoSelect™ 24-well migrasie toetsingseenheid gebruik is, is uitgevoer met *A. muthi-muthi* jel. Geen verbetering in migrasie in vergelyking met 'n onbehandelde kontrolegroep is egter waargeneem nie.

**Sleutelbegrippe:** Wondgenesing, *Aloe vera, Aloe ferox, Aloe muthi-muthi, Cyclopia,* heuningbos, krapwondtoets

## CONFERENCE PROCEDINGS

Fouché, M., Willers, C., Malherbe, C.J., Hamman, J.H. and Steenekamp, J.H. 2018. **Effects of selected plant materials on *in vitro* wound healing using the HaCaT cell culture model.** Oral presentation delivered at the First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST).

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## LIST OF ABBRIVIATIONS

| | |
|---|---|
| Acemannan | Acetylated mannan |
| BWAT | Bates-Jensen wound assessment tool |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| ECM | Extracellular matrix |
| FGF | Fibroblast growth factor |
| HaCaT | Human immortalised keratinocytes |
| HPEK | Human primary epidermal keratinocytes |
| HPLC-DAD | High performance liquid chromatography with diode-array detection |
| I3G | 3-β-D-glucopyranosyl iriflophenone |
| IDG | 3-β-D-glucopyranosyl-4-β-D-glucopyranosyl oxyiriflophenone |
| IL-1 | Interleukin-1 |
| M3G | 3-β-D-glucopyranosyl maclurin |
| MAPK | Mitogen-activated protein kinase |
| MMPs | Metalloproteinases |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MTT | Methyl thiazolyl tetrazolium |
| NEAA | Non-essential amino acids |
| NO | Nitric oxide |

| PBS | Phosphate buffered saline |
|---|---|
| PDGF | Platelet derived growth factor |
| PLGA | Poly(lactic-co-glycolic acid) |
| PVP | Polyvinyl-pyrrolidone |
| rhEGF | Recombinant-human epithelial growth factor |
| SD | Sprague-Dawley |
| TGF-β | Transforming growth factor-beta |
| TNF-α | Tumour necrosis factor-alpha |

# CHAPTER 1 INTRODUCTION AND PROBLEM STATEMENT

## 1.1 Introduction

Physical skin wounds can be categorised into five major groups, namely abrasions, incisions, lacerations, avulsions and puncture wounds (Anderson, 1997). Abrasions are caused by shear and inflict damage to the superficial and outermost layers of the skin (Riviello, 2010). Incisions are cuts into and through the skin and are caused by sharp objects (Anderson, 1997). Lacerations and avulsions are wounds where tissue is teared by shearing and tension forces. Avulsions differ from lacerations by being more severe, with parts of skin being removed completely in some cases. Puncture wounds are caused when objects penetrate the skin and underlying tissue (Anderson, 1997).

Wound healing is a process consisting mainly of four overlapping phases that begins as soon as an injury occurs (Wang *et al.,* 2018). The wound healing phases include the haemostasis phase, the inflammatory phase, the proliferation phase and the remodelling phase (Harper *et al*., 2014). One of the main mechanisms of wound healing in human skin is re-epithelialisation and not wound contraction, which occurs in rodents and other loose-skinned mammals that are often used in *in vivo* wound healing studies (Sullivan *et al.,* 2001). For *in vitro* wound healing studies, a model using the human immortalised keratinocyte cell line (HaCaT cells), represents a viable method to simulate wound healing, as it can serve as a representation of proliferative epidermal tissue (Boukamp *et al*., 1988; Fox *et al*., 2017; Lehmann *et al*., 1997).

The use of herbal wound healing remedies making use of aloe plant material has a history dating back to ancient times (Chen *et al*., 2012; Steenkamp & Stewart, 2007). *Aloe vera* is the most widely used and correspondingly, the most widely studied species in the *Aloe* genus (Hamman, 2008, Krishnan, 2006). *Aloe ferox* is an aloe species endemic to South Africa and is also used as a wound healing remedy among other applications, leading to its harvesting and processing becoming a multi-million Rand industry (Newton &Vaugh, 1996; Shackleton & Gambiza, 2007). Recently, a hybrid of *A. vera* and *A. ferox*, called *A. muthi-muthi* has been cultivated by means of forced cross-pollination.

Species of the genus *Cyclopia* are endemic to the fynbos biome of the Western and Eastern Cape provinces of South Africa (Joubert *et al*., 2008; Kokotkiewicz & Luczkiewicz, 2009). Colloquially known as honeybush, these plants are used to make herbal infusions or teas. Honeybush is a dietary source of various bioactive phytochemicals. The potential health promoting benefits of the use of *Cyclopia* have been attributed to the anti-oxidant capacity of its phytochemical constituents (Kamara *et al.,* 2003; Schulze *et al*., 2015). *C. intermedia* has also demonstrated inhibitory effects on tumour growth (Marnewick *et al*., 2005).

## 1.2 Wound healing scratch assay

The scratch assay (Figure 1.1) simulates wound healing within an *in vitro* setup, which involves culturing of an appropriate cell line. Cells are cultured to form monolayers on the bottoms of wells within cell culture plates. Scratches are induced into the cell monolayer with an object such as a 200 µl pipet tip to simulate wound gaps (Liang *et al*., 2007). The closure and closure rate of the scratches (or wound gaps) after treatment with a chemical compound or plant extract/material can then be compared to the closure of scratches induced in untreated wells, by capturing images of the wells periodically and measuring the closure with the aid of software.

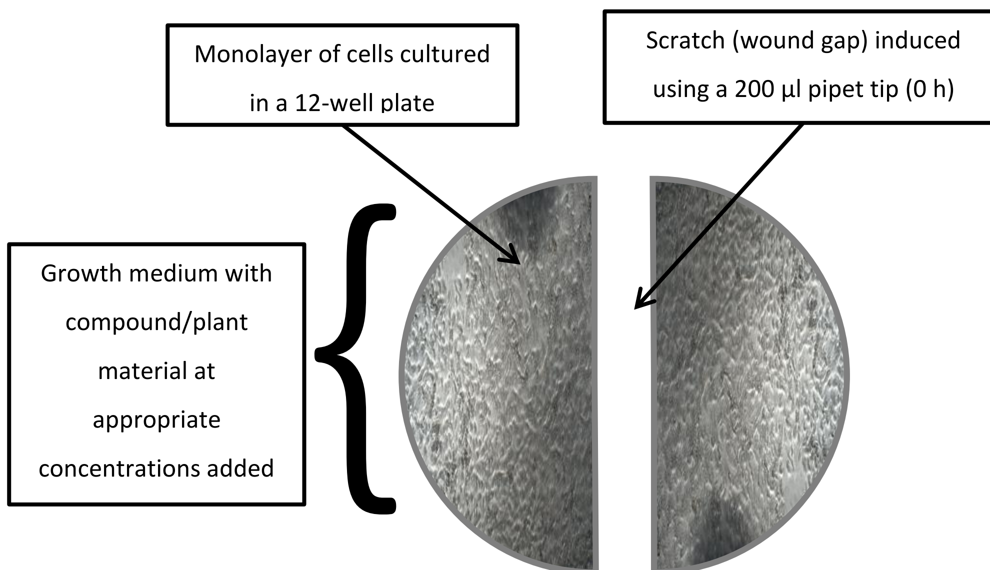


**Figure 1.1: Schematic illustration of an *in vitro* wound healing scratch assay setup**

## 1.3 *In vitro* cell migration assay

Cell migration is a key aspect during the wound healing process. Cell migration can be studied using a cell migration assay kit such as the CytoSelect™ 24-well cell migration assay (Cell biolabs, Inc.). The assay involves seeding cells into porous polycarbonate membrane inserts inside wells in a cell culture plate and incubating the cells for a predetermined time period. The migratory cells are quantified by staining the undersides of the insert membranes through which the cells migrated, thereafter extracting the stain absorbed into the cells and measuring the absorbance of the extracted stain solutions (Figure 1.2).

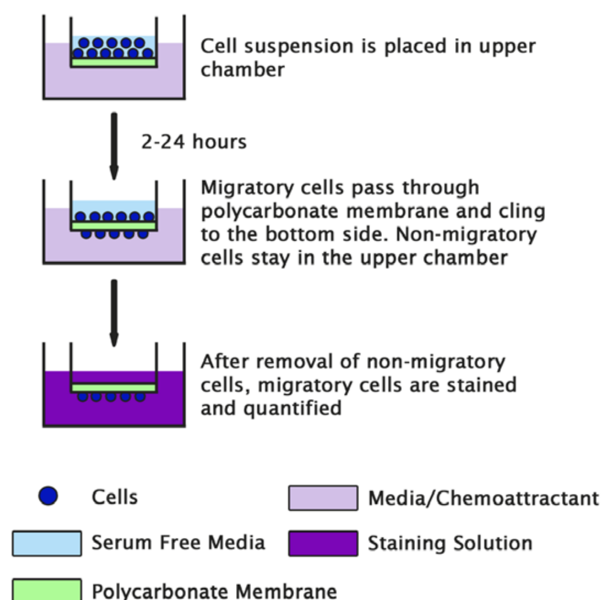


**Figure 1.2: Schematic illustration of the CytoSelect™ cell migration assay principle (Cell biolabs, Inc., 2017)**

### 1.4 Cytotoxicity

It is crucial that any therapeutic entity (e.g. plant extract/material) does not exhibit cytotoxicity in cells at concentrations where they exhibit biological activity. Therefore, cell viability needs to be assessed after the cells have been exposed to a series of concentrations of the investigative plant materials. The methyl thiazolyl tetrazolium (MTT) assay makes it possible to evaluate cell viability after exposure to the selected plant materials for 48 h (Berridge *et al*., 2005).

### 1.5 Problem statement

*Aloe* species have established wound healing effects *in vitro*. As *A. muthi-muthi* is a relatively recently cultivated hybrid, no experimental data on its wound healing potential has been published. Furthermore, although *Cyclopia* species are consumed for perceived health benefits, the wound healing characteristics of these plants remain unkown. Experiments testing the *in vitro* wound healing effects of both *A. muthi-muthi* and extracts of *C. genistoides* will contribute to the current body of knowledge on the subject of herbal wound remedies.

### 1.6 Research aim and objectives

The aim of this study was to investigate the *in vitro* wound healing effects of *A. muthi-muthi* gel and whole leaf materials, as well as crude extracts (including ethanolic and aqueous extracts) and chemical fractions of *C. genistoides.* The research question to be answered was if *A. muthi-muthi* has wound healing effects similar to that reported for other aloe species, especially

*A. vera* and *A. ferox* from which it was derived. It was also necessary to determine whether extracts from *Cyclopia* demonstrates any *in vitro* wound healing effects.

The objectives of this study were:

- To culture HaCaT cells in monolayers on the bottoms of the wells of 12-well cell culture plates.
- To measure the cell viability of HaCaT cells after exposure to the selected plant extracts/materials (including honeybush extracts and fractions, as well as *A. muthi-muthi* gel and whole leaf extracts) in order to determine their cytotoxicity potential.
- To determine *in vitro* wound healing effects of the selected plant extracts/materials by conducting a scratch assay and measuring the wound closure, as well as the closure rate during exposure to the selected plant extracts/materials.
- To determine cell migration using a CytoSelect™ 24-Well cell migration assay kit after treatment with the plant material that displayed the most notable effects on wound closure and closure rate.

### 1.7 Outline of dissertation

This first chapter serves as an introduction to the study and outlines the aims and objectives, as stated in Section 1.5. Chapter 2 is a literature overview on the wound healing process, wound treatments, including traditional remedies and a more detailed description of the botany of *Aloe* and *Cyclopia* species. Models to investigate wound healing (both *in vivo* and *in vitro*) are also discussed in the literature overview chapter. Chapter 3 is an article manuscript which will be formatted according to the author guidelines of *Planta Medica* (international scientific journal to which the manuscript will be submitted), titled: *Wound healing effects of selected plant materials: in vitro investigations using the HaCaT cell culture model*. For the purposes of this dissertation, the style was in line with the rest of the chapters. Chapter 4 contains the conclusion and future prospects for further research. The appendices provide further information on the conference proceedings, the author guidelines for *Planta Medica* and detailed data obtained from the experiments.

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# CHAPTER 2 OVERVIEW OF WOUND HEALING, TREATMENT OF WOUNDS AND WOUND HEALING MODELS

## 2.1 Introduction

The following chapter is a literature overview on wound healing. Different wound types and the physiological processes of wound healing are discussed, along with various treatments of wounds, including both traditional remedies as well as modern pharmaceutical products. This chapter also provides a brief overview of the botany of the plant genera used in this study. Furthermore, both *in vitro* and *in vivo* wound healing models useful for wound healing experiments are considered.

## 2.2 The process of skin wound formation and healing

### 2.2.1 Types of wounds

A wound can be defined as a disruption or injury to the skin (Orgill & Blanco, 2009). Wounds can be categorised according to their causes, as well as how they impact the tissues where they occur. Physical wounds (excluding burn wounds) are commonly divided into five categories, which include abrasions, incisions, lacerations, avulsions and puncture wounds (Anderson, 1997).

Abrasions occur when the skin is scraped on the surface and the superficial layers of the skin are damaged or removed by shear. Abrasions do not penetrate the dermis (Riviello, 2010). Incisions are wounds caused by sharp objects that cut through the skin (Anderson, 1997). Incisions heal quicker and the risk of infection is lower than with other wounds, such as lacerations or avulsions, due to lack of irregular edges and improved blood flow associated with these wounds (Cooper & Gosnell, 2015). Lacerations occur when skin is torn by a combination of tension and shearing forces (Anderson, 1997). Bleeding is often more profuse with lacerations and destruction of tissue is more likely to occur (Cooper & Gosnell, 2015). Avulsions, like lacerations, occur when skin is torn, but they are more severe as the skin is completely separated from the underlying tissue (Anderson, 1997). With an avulsion, a section of skin may be removed completely or still be left attached as a loose flap (Cooper & Gosnell, 2015). Puncture wounds are caused by the penetration of the skin and underlying tissues. Even when damage to the superficial layers of the skin appears minimal, puncture wounds can be very severe depending on the depth of penetration. Puncture wounds also pose some complications for treatment, as the penetrating object may be embedded in the wound (Anderson, 1997).

Another wound category is burn wounds. Burn wounds can further be divided into different sub-categories corresponding to their causes, including thermal burns, chemical burns and electrical burns (Koh *et al.*, 2017). As the nomenclature implies, thermal burns are caused by heat-generating objects. Thermal burns may be caused by direct contact with flames or hot objects or indirectly, e.g. heat generated by flames or hot objects (McCullogh & Kloth, 2010). The part of the wound that is exposed to the most heat will correspondingly be the site of greatest damage. Protein denaturation, which occurs at temperatures exceeding 41°C, is an additional consequence of burn wounds and extensive denaturation, due to excessive heat exposure will lead to greater severity in tissue degradation (Rowan *et al.*, 2015). Chemical burns are caused when substances, especially acids and bases, cause burn injuries. Even common household chemicals can cause significant burn injuries. Chemical burns are also an occupational hazard in various industries that make use of chemicals, from electroplating and semiconductor manufacturing to wastewater treatment (Koh *et al.*, 2017). Electrical burns are burn wounds caused by exposure to electricity. How an electrical current affects the body are determined by factors such as the amount of current, type of current (alternating- or direct current), path that the current takes through the body, duration of exposure to the current, bodily resistance and voltage (Li *et al.*, 2017).

Burn wound severity is often characterised according to the depth or thickness of the burn. Typically, superficial burns that render the skin red, sensitive and without blisters are called first-degree burns. Second degree burns are burns that cause significant damage to the skin and also damage the skin's microcirculation (Xu, 2004). Blisters may also develop with second degree burns, but not necessarily. Third degree burns are the thickest and most damaging types of burn wounds. They may be white and pliable or charred and dark coloured or even bright red (McCullogh & Kloth, 2010).

Crucial to providing an optimal environment for the healing of burn wounds, as with all other wounds, is prompt treatment. Initial management should include proper first aid, applying appropriate dressings and of special importance in the case of burns - the management of swelling (Kenworthy *et al.*, 2018).

### 2.2.2 The process of wound healing

The process involved with wound healing is a complex sequence of phases that overlap. These events are initiated as soon as injury occurs (Wang *et al.,* 2018). The process of wound healing can be divided into four phases: the haemostasis phase, the inflammatory phase, the proliferation phase and the remodelling phase (Harper *et al.*, 2014; Kibe *et al.,* 2017; Velnar *et al.*, 2009). After an injury such as a surgical incision has occurred, vascular damage at the

micro- or macrovascular level initiates the haemostasis phase (Harper *et al.,* 2014). The haemostasis phase, largely involves the coagulation of blood (Kibe *et al.*, 2017). Exposed sub-endothelial tissue, collagen and tissue factor initiates platelet aggregation. Chemokines and growth factors are released and degranulation occurs (Wang *et al.,* 2018). Degranulation is an important protective function where degradative enzymes and receptors involved in the recognition of pathogens are released. This process works as a defensive mechanism against infection (Babin *et al.*, 2013). The first phase serves an initial protective function, primarily preventing excessive blood loss, but also providing protection against infectious pathogens. Coagulation of blood protects the vascular system and prevents organs from becoming blood-deprived (Velnar et al., 2009). Shortly after injury, the constriction of blood vessels leads to tissue hypoxia and acidosis, which promotes the production of nitric oxide (NO), adenosine and other vasoactive metabolites. These metabolites dilate blood vessels as a reflexive mechanism which increases permeability for inflammatory agents (Harper *et al.,* 2014).

The inflammatory phase follows haemostasis. It can be divided into early and late inflammatory phases. Starting during the latter part of haemostasis, the early response of inflammation activates a cascade of events. Neutrophils are the first cells to infiltrate the wound site in order to prevent infection and start the process of phagocytosis in order to rid the wound of debris and bacteria (Wang *et al.,* 2018). Monocytes soon follow the neutrophils, activating within one to two days to become macrophages (Orgill & Blanco, 2009). The macrophages are drawn to the wound site by transforming growth factor-beta (TGF-β), which also stimulates the macrophages to produce cytokines. Cytokines are a group of heterogeneous proteins with diverse functions, including immunoregulation, cell proliferation and cell differentiation, induction of apoptosis and proinflammatory activity (Katzung *et al.*, 2012). Some of the cytokines involved in the wound healing process include fibroblast growth factor (FGF), tumour necrosis factor-alpha (TNF-α), platelet derived growth factor (PDGF) and interleukin-1 (IL-1) (Beldon, 2010). The late inflammatory response, which occurs between two and three days after the injury, is characterised by the appearance of these macrophages which continue the process of phagocytosis initiated in the early stage of inflammation (Velnar *et al.,* 2009).

The third phase, known as the proliferative phase, initiates the first step towards rebuilding damaged tissue. Simultaneous processes in this reparative stage include: angiogenesis, granulation tissue formation, reepithelialisation and wound retraction (Harper *et al.*, 2014). Angiogenesis is the process where new blood vessels are formed. This is a very important process, considering the oxygen and nutrient requirements of the proliferating fibroblasts and endothelial cells (Nawaz & Bentley, 2011). Granulation tissue formation occurs as PDGF and TGF-β released by fibroblasts induce proliferation of both fibroblasts and epithelium, leading to

the deposition of collagen, adhesive glycoproteins and proteoglycans. These deposited components together form the extracellular matrix (ECM) (Nawaz & Bentley, 2011). Reepithelialisation is the forming of new epithelium over the wound and involves both the proliferation and migration of keratinocytes at the peripheral area of the wound site (Santoro & Gaudino, 2005).

The fourth and final stage, remodelling, is characterised by the formation of normal epithelium and the scar tissue becoming mature (Harper *et al.*, 2014). The remodelling of wounded tissue increases the tensile strength thereof as fibroblasts maintain a balance between synthesis of new tissue and degradation of wounded tissue by means of enzymes known as metalloproteinases (MMPs). The remodelling process is slow and may take longer than a year (Beldon, 2010).

## 2.3 Treatment of wounds

Traditional remedies have been used to treat wounds for millennia and even in modern times many such remedies are still used around the world. Correspondingly, modern developments in products such as wound dressings and antiseptics have been developed to improve wound healing outcomes.

### 2.3.1 Chemical- and pharmacological substances used in wound treatment

A wide variety of products, substances and dosage forms are used in the treatment of wounds such as alginates, antimicrobials, foams and hydrocolloids, among others (Hess, 2012). Antimicrobials are often applied as part of wound dressings to deliver antimicrobial action topically (Hess, 2012). Perhaps the most commonly used topical antimicrobials are silver, iodine and certain antibiotics.

Silver has well-established antimicrobial properties and is used in topical wound care applications (Fong & Wood, 2006). Products like silver-sulfadiazine combine both the antimicrobial properties of silver with an antibiotic (Murphy & Evans, 2012). Murphy and Evans (2012) noted, however, that complications such as a higher rate of resistance compared to silver nitrate, impaired reepithelialisation and pseudo-eschar formation render silver-sulfadiazine less than ideal for wound management. Nanocrystalline silver is a more recent development in silver-based wound dressings and has shown to be clinically effective and offer benefits over silver-sulfadiazine dressings such as sustained release and less frequent dressing changes (Fong & Wood, 2006; Murphy & Evans, 2012). It should be noted, however, that silver nanoparticles have been found to exhibit long lasting anti-proliferative effects on keratinocytes *in vitro* (Zanette *et al.,* 2011).

Iodine-based treatments are commonly used for topical wound disinfection. In a systematic review evaluating the benefit and harm of iodine in wound care, Vermeulen *et al.* (2010) noted that iodine is probably the most well-known antiseptic, being in use for more than a century. However, its use has been questioned as concerns were raised regarding allergic reactions, poor penetration leading to poor efficacy and poor tissue regeneration due to toxicity (Brånemark *et al.,* 1966; Hagedorn *et al.*, 1995; Lineaweaver *et al.*, 1985; Rodeheaver *et al.*, 1982). More recent research has, however, shed more light on the advantages of iodine in wound care. Vogt *et al.* (2006) found significant reduction in skin graft loss with burn wounds treated with a polyvinyl-pyrrolidone (PVP)-iodine hydrogel in a hydrosome formulation. This study built on findings which investigated the use of PVP-iodine in a liposomal hydrogel formulation. PVP-iodine showed improved reepithelialisation and also significant reduction in skin graft loss compared to a control consisting of chlorhexidine gauze (Vogt *et al.,* 2001).

Research studies supported the use of topical antibiotics in the treatment of clean wounds as prophylactics against bacterial infection (Diehr *et al.*, 2007). For minor contaminated wounds, triple antibiotic ointments (combining neomycin sulphate, bacitracin zinc and polymyxin B sulphate), topical silver sulfadiazine or topical bacitracin zinc can significantly reduce infection rates (Dire *et al.,* 1995). Theunissen *et al.* (2016) found improved wound healing with topical antibiotics in an *in vivo* wound healing study on domestic pigs. The study compared 5% povidone-iodine cream, 1% silver-sulfadiazine, 2% mupirocin, and 1% silver-sulfadiazine combined with 1 mg/100 g recombinant-human epithelial growth factor (rhEGF) with an untreated control.

Along with modern medicinal advancements in wound treatments, various traditional remedies have been used for millennia around the world. Research into many of these remedies has also provided vindication for their use, as well as provided more options for wound therapy in general.

### 2.3.2 Traditional remedies used in wound treatment

Various traditional remedies have been and are still used around the world for the treatment of wounds. Most of these traditional wound remedies are of plant origin. Scarlet pimpernel (*Anagallis arvensis* L.) and blue pimpernel (*Anagallis foemina* Mill.) are plant species that have traditionally been used as wound healing remedies in Navarra, Spain. Extracts from both of these plant species have been found to exhibit anti-inflammatory and bacteriostatic properties *in vitro* (López *et al.*, 2011). At least one of four saponins extracted from *A. arvensis* L. has also demonstrated *in vitro* antifungal activity against *Candida albicans*, which supports the traditional use of this plant in Argentina as an antifungal remedy (Soberón *et al.,* 2017).

*Bulbine* plant species are used traditionally as skin remedies in Southern Africa (Pather *et al.*, 2011). In a study which examined both excisional and incisional wounds on domestic pigs, *Bulbine frutescens* and *Bulbine natalensis* exhibited significant improvement in wound contraction compared to an untreated control. Significant improvement in collagen, protein and deoxyribonucleic acid (DNA) content was also found in wounds treated with both of these *Bulbine* species (Pather *et al.*, 2011).

Honey is another naturally occurring substance traditionally used for the treatment of wounds since ancient times. This is due to its bacteriostatic and bactericidal effects (Lusby et al., 2002). Lusby *et al.* (2002) noted that the therapeutic use of honey in the form of an ointment has been recorded in the Smith papyrus of 1700 B.C. Honey was mixed with fat in a 1:2 ratio and then applied to a wound. Active Manuka honey from New Zealand and Medihoney® from Australia are currently both approved for therapeutic use in their raw form. Both of these therapeutic honeys are derived from the nectar of tea trees (*Leptospermum* spp.) (Lusby *et al.,* 2002). Various honeys, including active Manuka honey and Medihoney® have shown *in vitro* bacteriostatic effects against a range of microorganisms. In an *in vitro* study, Lusby *et al.* (2005) investigated the antibacterial effects of Medihoney®, active Manuka honey (both derived from *Leptospermum* spp.) as well as honeys derived from red stringy bark (*Eucalyptus macrorhyncha*), lavender (*Lavandula x allardii*) and Paterson's curse (*Echium plantagineum*). All of the honeys tested demonstrated bacteriostatic effects against *Alcaligenes faecalis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacterium phlei*, *Salmonella california*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus and Staphylococcus epidermidis*. The honeys, however, did not show bacteriostatic activity against *Serratia marcescens* or inhibit the growth of *Candida albicans*. Synergistic effects between Manuka honey in combination with tetracycline, imipenem, as well as with mupirocin have been observed *in vitro* against a methicillin-resistant *Staphylococcus aureus* (MRSA) strain (Jenkins & Cooper, 2012).

Phytochemicals that are found in traditional wound healing remedies around the world commonly have anti-oxidant or anti-inflammatory properties (Shah & Amini-Nik, 2017). Turmeric and plants of the *Terminalia* genus are examples of such remedies used in Asian traditional wound healing remedies (Shah & Amini-Nik, 2017). Curcumin has been found to improve the contraction rate of excision wounds in mice, which had been exposed to $\gamma$-radiation. Increased fibroblast and vascular densities were observed along with increased deposition of collagen and increased formation of DNA, NO and hexosamine (Jagetia *et al.*, 2004).

Herbal infusions, commonly called "teas" have been traditionally consumed in South Africa, at least partly for health benefits (Joubert *et al.,* 2008). Amongst these are rooibos (*Aspalathus*

*linearis*), honeybush (*Cyclopia* spp.) and "bush tea" (*Athrixia phylicoides*), with the latter having been used for the treatment of boils, acne and infected wounds (Joubert *et al.*, 2008).

Honeybush (*Cyclopia* species) has been found to be a dietary source of bioactive phytochemicals such as polyphenols, xanthones, benzophenones and flavones like hesperidin and dihydrochalcones, when consumed as a herbal infusion (Kamara *et al.,* 2003; Schulze *et al.*, 2015). Antioxidant activity may be one of the main reasons that the consumption of herbal extracts rich in these phytochemicals has promising health benefits. In a review article on the bioactivity of both rooibos and honeybush, McKay and Blumberg (2007) mentioned numerous studies that evaluated the antioxidant capacity and antimutagenic properties of these plants. Kamara *et al.* (2003) evaluated the polyphenol content of honeybush (*Cyclopia intermedia*) extracts. A significant variety of flavonoids and other polyphenols were present in tea brewed from *C. intermedia* and it was concluded that the claimed health-promoting effects of honeybush may be attributed to the antioxidant activity and low caffeine content thereof (Kamara *et al.*, 2003). *C. intermedia* and other herbal extracts' potential to inhibit tumour growth in mice has been evaluated *in vivo* with promising results. The inhibition of tumour growth has been attributed to variations in composition of flavonol/proanthocyanidin and flavonol/flavone, as well as other non-phenolic compounds present in these extracts (Marnewick *et al.,* 2005). Both *C. maculata* and *C. subternata* have been found to inhibit adipogenesis in 3T3-L1 adipocytes in an *in vitro* study, with reduced accumulation of intracellular fat and triglycerides having been observed. This suggested potential for honeybush as a weight-loss aid (Dudhia *et al.*, 2013). Fermented and unfermented extracts of *C. intermedia* have been found to exhibit some, albeit weak antimicrobial activity (Dube *et al.,* 2017).

The use of *Aloe* species for medicinal applications dates back thousands of years. Steenkamp and Stewart (2007) noted that the botanist and physician, Dioscorides, described the use of *Aloe* for the treatment of wounds as early as the first century A.D. *Aloe* species such as *Aloe vera* (also known as *Aloe barbadensis* Miller)*, Aloe ferox* and *Aloe marlothii* have been shown to increase the rate of wound healing in an *in vitro* wound healing model using human immortalised keratinocytes (HaCaT cell line) (Fox *et al.,* 2017). Limited *in vitro* cytotoxic effects on keratinocytes and on fibroblasts from exposure to plant material from different species of *Aloe* have been observed when methyl thiazolyl tetrazolium- (MTT) assays were conducted (Fox *et al.,* 2017; Ghayempour *et al.,* 2016). Beneficial effects of *Aloe vera* on wound healing have also been observed in an *in vitro* model using human primary epidermal keratinocytes (HPEK) and a human skin equivalent model. Increased expression of integrin receptors (β1, α6 and β4) and E-cadherin was observed in HPEK treated with *Aloe vera* (Moriyama *et al.,* 2016).

Phytochemicals in *Aloe vera* leaves cover a broad range of polysaccharides, anthraquinones, chromones, enzymes and vitamins. Among the polysaccharides present in the leaf gel of *Aloe vera*, partially acetylated mannan (acemannan) has been identified as the primary polysaccharide responsible for biological activity of the gel material (Hamman, 2008). The secondary compound, aloesin, an aromatic C-glucosylated 5-methylchromone that occurs in *Aloe vera*, has been found to improve skin wound healing in both *in vitro* models using keratinocyte cell culture models and *in vivo* using a mouse model. The modulation of signalling pathways such as and MAPK (mitogen-activated protein kinase) are considered the mechanism by which aloesin accelerated the wound healing process (Wahedi *et al*., 2017).

Research into the wound healing properties of *Aloe* has also ventured beyond human health science and into the realm of veterinary wound healing studies. Recently, a comparative study was conducted which compared the wound healing effects of both the juice and gel of *Aloe vera* leaves with silver-sulfadiazine on 13 dog- and 3 cat patients who had suffered 1 or more traumatic lesions to the skin. The treatment with *A. vera* juice and gel showed improved lesion contraction, reduced healing-time and decreased severity when evaluated with the Bates-Jensen wound assessment tool (BWAT) method (Drudi *et al*., 2018).

As a result of the wound healing properties of *A. vera*, it has been incorporated into novel dosage forms and wound dressings: The development of- and investigation into nanofibrous wound dressings is a good example of this (Garcia-Orue *et al.,* 2016). In a study involving male mice, Garcia-Orue *et al.* (2016) found significantly increased wound area reduction with poly(lactic-co-glycolic acid) (PLGA) nanofibre dressings combined with *A. vera* and rhEGF, as compared to the effects of these substances individually or combinations of PLGA and rhEGF without *A. vera*. *Aloe vera* has also been found to exhibit *in vitro* antibacterial and antifungal activity: *A. vera* was incorporated into a nano-emulsion, which encapsulated the *A. vera* in tragacanth gum, forming nanocapsules, which were added to concentrated suspensions of *E. coli, S. Aureus* and *C. albicans* using an established shake flask method. A significant inhibition in the growth of treated cultures was observed compared to an untreated control (Ghayempour *et al.,* 2016).

## 2.4 Botany of *Aloe* spp. and *Cyclopia* spp.

Since traditional wound healing remedies are largely sourced from plants, the botany of these plants can provide useful insight. An example of a useful application of botany in traditional medicine is the proper identification of the plant species, the chemical composition of the plant material and other important aspects such as which part of the plant contains the bioactive molecule.

### 2.4.1 *Aloe vera*

*Aloe* is a genus of plants in the family Asphodelaceae. It has a wide geographic distribution, ranging from tropical to Southern Africa, the island of Madagascar and from Jordan to the Arabian Peninsula (Govaerts & Newton, 2018a). *Aloe vera* (Figure 2.1) is a succulent, which is considered originally native to the South Western parts of the Arabian Peninsula (Govaerts & Newton, 2018b). The plant's thick, green leaves are tapered and thorny and contain a clear gel (Kumar *et al.,* 2017). The leaf consists of two primary parts, namely the outer rind and the inner pulp or gel. The unprocessed pulp from *A. vera* leaves consists mainly of water, while the remaining 0.5 – 1.0% w/w of solid material contains a variety of compounds, including polysaccharides, minerals, enzymes, phenolic compounds, organic acids, as well as both water-soluble and lipid-soluble vitamins (Hamman, 2008).

*Aloe vera* is the most important of all the *Aloe* species in terms of cultivation for commercial interests and the processing thereof for a wide array of health-related applications has become a worldwide industry (Hamman, 2008). It is also the most widely studied *Aloe* species, and probably the most researched medicinal plant, having been subjected to various cell-culture based-, animal- and human studies (Krishnan, 2006).

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

### 2.4.2 Aloe ferox

*Aloe ferox* (Figure 2.2), also known as bitter aloe or Cape aloe, is indigenous to Southern Africa, with a geographic range extending from the Cape region to the southern areas of Kwa-Zulu Natal and parts of Lesotho (Govaerts & Newton, 2018c; Chen *et al.*, 2012). The plant has thorny leaves with distinctive reddish spines and erect racemes of mostly red, orange or yellow

flowers (Chen *et al*., 2012). The bitter latex or exudate from *A. ferox*, which contains aloe emodin, is commonly used for laxative purposes (Grace *et al*., 2008). Presumably like *A. vera*, the use of *A. ferox* for medicinal purposes also dates back to ancient times, as depictions of this plant have been found in San rock paintings (Chen *et al*., 2012). The harvesting of *A. ferox* has also developed into a multimillion-rand industry in South Africa, with approximately 400 tons of bitters being produced per year as far back as 1996 with a value to local harvesters alone being approximately R4 million, according to a report by Newton & Vaughn (1996). Ten years later, it was estimated that the industry's value to local harvesters had grown to between R12- and R15 million and if total retail mark-up were brought into account, the total value of the *A. ferox* industry in South Africa was estimated to be as high as R150 million if not greater (Shackleton & Gambiza, 2007).

**Figure 2.2:** ***Aloe ferox*. Note the red racemes (Aubrey, 2001)**

### 2.4.3 *Aloe muthi-muthi*

*Aloe muthi-muthi* (Figure 2.3) is a hybrid aloe species that was formed by means of forced cross-pollination of *Aloe vera* and *Aloe ferox*. This aloe species was first cultivated by Mr Jaap Viljoen and Hannes Viljoen of Rooiklip nursery in Swellendam (South Africa). Its thorny leaves resemble those of *A. vera* and it exhibits erect racemes of yellow flowers. The botany of both *A. vera* and *A. ferox* are therefore important when considering *A. muthi-muthi's* characteristics.

**Figure 2.3: Photo of *Aloe muthi-muthi* plant supplied by Rooiklip nursery**

### 2.4.4 *Cyclopia* species

*Cyclopia* species (Figure 2.4), colloquially known as honeybush, are part of the Fabaceae plant family. These species grow in the mountainous and coastal regions of the Western- and Eastern Cape provinces of South Africa, also known as the fynbos shrubland (Kokotkiewicz & Luczkiewicz, 2009; Joubert *et al.*, 2008). The woody-stemmed bushes can grow between 1.5 and 3.0 m high, depending on the species. The shapes and sizes of the leaves also differ between species. Tri-foliate leaves and flowers with indented calyx are distinctive characteristics of this genus (Joubert *et al.,* 2008). The flowers' honey-like scent is the most likely reason for the colloquial name, honeybush. *Cyclopia intermedia, Cyclopia subternata, Cyclopia sessiliflora* and *Cyclopia genistoides* are the major species of commercial interest, with mainly *C. subternata* and *C. genistoides* being commercially cultivated and supply being supplemented by the wild-harvesting of other species such as *C. genistoides* to cater to the demand for honeybush (Joubert *et al.,* 2008; 2011).

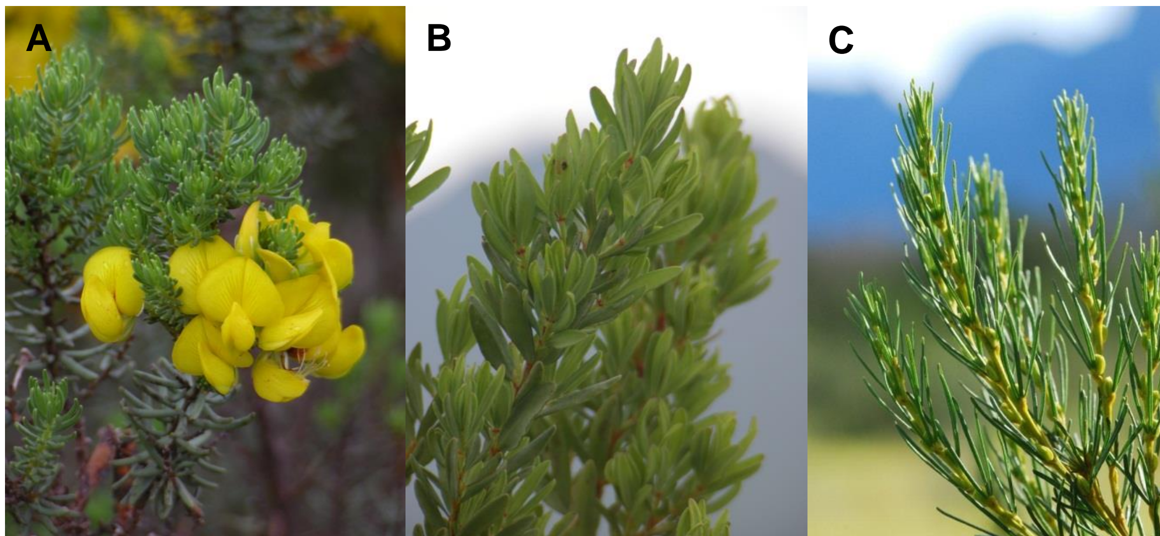


**Figure 2.4: A) *Cyclopia genistoides* flowers (SAHTA, 2018a). B) *Cyclopia intermedia* branch (SAHTA, 2018b). C) *Cyclopia subternata* branch with flower buds (SAHTA, 2018c).**

### 2.5 Models to investigate wound healing

In order to investigate the wound healing effects of plant materials, an appropriate model has to be selected to represent the process of wound healing accurately. Different wound models are used in research, each having respective benefits and disadvantages.

Excluding clinical case studies, wound healing can be investigated by using *in vivo* or *in vitro* models. A substantial amount of *in vivo* and *in vitro* wound healing models have been developed as a response to the development of novel wound treatments (Martin *et al.,* 2016). Both *in vivo* and *in vitro* models present distinctive advantages and disadvantages. The choice of which model to use depends on the specific experimental requirements of the research in question.

#### 2.5.1 *In vivo models*

*In vivo* wound healing models involve the use of live subjects such as test animals. Wounds are deliberately inflicted for the purpose of investigating them in *in vivo* wound healing experiments. *In vivo* wound healing studies are often performed on small, loose-skinned mammals. For example, the effects of diclofenac on wounds in Wistar rats have been investigated. These wounds were made with a metallic punch, which ensured consistency in the size of the wounds (Da Silva Costa *et al.,* 2014). Du *et al.* (2012) tested a multifunctional *in situ*-forming hydrogels on different types of animals: Sprague-Dawley (SD) rats and New Zealand rabbits. Wounds

were inflicted in the rabbits and rats by cutting consistently sized incisions into the ears of the rabbits and backs of the rats.

Sullivan *et al.* (2001) suggested the pig as a preferred wound healing model over smaller mammals like mice and rats. It was argued that the porcine model is superior based on the anatomical and physiological differences between human skin and the skin of small mammals, whereas strong similarities exist between human skin and pig skin. Unlike human skin, the skin of small mammals, like that of rodents, heals primarily through wound contraction and not reepithelialisation, whereas pig skin shows greater similarity with human skin in terms of its primary wound healing mechanism (Sullivan *et al.,* 2001). The porcine model has also been used in research on burn wounds, specifically in investigation into dextran-based hydrogels as synthetic burn wound treatments. Third degree burn wounds were inflicted on pigs with a custom- made device, with a metal heat source and pressure units for consistent wounding. Biopsies of both treated and untreated wounds were taken after 24 and 48 h and it was found that dextran-based hydrogels delivered notable improved burn wound healing results (Shen *et al.,* 2015*).*

Although *in vivo* wound healing models may offer realistic wound healing data and even reasonably accurate representations of wound healing in human skin, they are ethically questionable as they often involve the deliberate infliction of injury to live animals. This, along with the cost of feeding and housing test animals in acceptable living conditions, make *in vivo* testing unacceptable if they cannot be properly justified and proper alternative methods or models are available.

### 2.5.2 *In vitro models*

*In vitro* wound healing models are a representation of the wound healing process or often only a part thereof, as they do not involve live test-subjects. They can therefore be referred to as wound simulation models. Ethically speaking, *in vitro* models offer better methods to investigate wound healing as no deliberate infliction of wounds in test animals is conducted. *In vitro* wound healing assays can be done using cell cultures of fibroblasts and keratinocytes to simulate wounded epithelial tissue (Fox *et al.,* 2017; Mazumder *et al*., 2016; Walter *et al.,* 2010) such as scratch assays, zone-exclusion assays and migration- and invasion assays.

#### 2.5.2.1 The HaCaT cell line

Human immortalised keratinocytes (HaCaT cells) are a line of cells originally obtained from human epidermal tissue (Boukamp *et al.*, 1988). The cell line can be cultured for numerous amounts of passages, even as high as 140 passages, without showing signs of complications

(Boukamp *et al.,* 1988). It exhibits aneuploidy and shows alterations in chromosomal sequence, but the chromosomal differences have not been found to cause major defects in cell differentiation (Boukamp *et al.,* 1988). These cells have been proposed as an *in vitro* model to study vitamin $D_3$ metabolism in human skin (Lehmann, 1997). HaCaT cells can be used as an *in vitro* model for very proliferative epidermal tissue (Lehmann, 1997). This makes them ideal for use in *in vitro* wound healing studies. Walter *et al.* (2010) found that mesenchymal stem cell-conditioned medium accelerated wound healing in both fibroblasts and HaCaT cells. Mazumder *et al.* (2016) and Fox *et al.* (2017) used HaCaT cells to simulate wound healing *in vitro* using kits for both zone-exclusion assays and migration- and invasion assays. Mazumder *et al.* (2016) found that a sinigrin-phytosome complex improved *in vitro* wound healing effects on HaCaT cells. Fox *et al.* (2017) demonstrated the *in vitro* wound healing effects of *Aloe vera*, *Aloe ferox* and *Aloe marlothii* on HaCaT cells.

#### 2.5.2.2 Methods used for *in vitro* wound healing studies

Wounds can be simulated in *in vitro* cell culture by means of zone exclusion. Kits that use inserts in cell culture wells were employed to provide a zone where cells cannot grow during the initial seeding and culturing (Fox et al., 2017; Mazumder *et al.,* 2016) When the insert is removed from the seeded cells, the open gap left by it, represents the wound. The rate of gap closure can then be used to evaluate cell migration and regrowth, which represents wound healing *in vitro* (Cell Biolabs Inc., 2017a). A benefit of this method is the consistency in the size of the gaps left by the inserts. A disadvantage of using zone exclusion-kits is the substantial cost of purchasing them, especially if experiments with a high number of replicates are conducted.

Another, more cost-effective, method to simulate wound healing is by means of a scratch assay. With this method, cells are seeded into cell culture well plates and monolayers are allowed to form on the bottom of the wells. After monolayer formation, a scratch is induced in the cell monolayer of each well. As with the zone exclusion method, the rate of gap closure of this scratch can be used to evaluate wound healing (Liang *et al.,* 2007). This method has been used on both keratinocytes and fibroblasts to evaluate *in vitro* wound healing effects of various substances (Chaudhary *et al.*, 2015; Walter *et al.,* 2010).

As wound healing involves the movement of cells to the affected area, migration- and invasion assays can provide more information into how experimental variables affect cellular migration. Kits, like the CytoSelect™ 24-well cell migration and invasion assay are used to determine the migration of cells using wells with specialised inserts (Cell Biolabs, 2017b). In the CytoSelect™ migration assay, these inserts form an upper chamber in the well. The insert's bottom consists

of a polycarbonate membrane with pores. Migratory cells pass through this membrane and cling to the bottom of the membrane of the upper chamber. The non-migratory cells are then removed, and the migratory cells clinging to the bottom of the chamber can be quantified (Cell Biolabs, 2017b). The CytoSelect™ invasion assay works on the same principle as the migration assay, with the exception that instead of a polycarbonate membrane, the bottoms of the inserts consist of a protein basement membrane and invasive cells degrade the proteins of this basement membrane, allowing them to pass through and cling to the outside of the insert (Cell Biolabs, 2017b).

### 2.6 Summary

Wound healing is physiologically complicated and involves multiple processes such as haemostasis, inflammation, cell-migration and reepithelialisation (Harper *et al.*, 2014; Kibe *et al.,* 2017; Velnar et al., 2009; Wang *et al.,* 2018). It is therefore important to consider all the mechanisms involved when investigating potential wound healing remedies. Reepithelialisation has been singled out as the primary mechanism of wound healing in humans, unlike that of loose skinned mammals, whose wounds heal primarily by wound contraction (Sullivan *et al.*, 2001). Differences like these are important to consider when choosing a model to investigate or simulate the wound healing process.

For thousands of years, mankind has relied on natural, mostly plant-based remedies for wound healing. Investigation into the wound healing effects of treatment with such remedies is essential, either to empirically determine how significant these effects are or how they can be administered for most optimal effects. *Aloe vera* and *Aloe ferox* species are some of the most prominent herbal wound healing remedies, and have been since ancient times (Chen *et al.,* 2012; Hamman, 2008; Steenkamp & Stewart, 2007). The wound healing potential of these plants have also been observed in *in vitro* and *in vivo* experiments (Drudi *et al.,* 2018; Fox *et al.,* 2017; Garcia-Orue *et al.*, 2016; Wahedi *et al.,* 2017).

For *in vitro* studies, cell culture-based models using both fibroblasts and keratinocytes are often used to simulate wound healing (Chaudhary *et al.*, 2015; Fox *et al.,* 2017; Mazumder *et al.,* 2016; Walter *et al.,* 2010). The HaCaT cell-line is able to undergo multiple passages and is a good representation of proliferative epidermal tissue, which makes it ideal for *in vitro* wound healing models (Boukamp *et al.*, 1988; Lehmann, 1997).

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# CHAPTER 3 ARTICLE

This chapter has been written in article format. For the purposes of this dissertation, the style (specifically the figures and tables being included within the body of text), with the exception of numbered headings, was kept in line with the rest of the dissertation, but the article will be submitted according to the author guidelines of the journal (Appendix B).

# Wound healing effects of selected plant materials: *in vitro* investigations using the HaCaT cell culture model

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**Abstract**

The traditional use of *Aloe* spp. for the purpose of wound healing has a long history and is wide spread internationally. Recently, a hybrid aloe plant (*Aloe muthi-muthi*) has been cultivated by cross pollination between *A. vera* and *A. ferox*. The *A. muthi-muthi* plant has not yet been investigated for medicinal properties and provides an opportunity for potential biological activity including wound healing. Herbal teas are known to contain anti-oxidants that provide certain health benefits. Honeybush tea is derived from different *Cyclopia* species and has been used traditionally for treatment of different ailments and also as a beverage. The aim of this study was to investigate the *in vitro* wound healing effects of both *A. muthi-muthi* leaf materials and extracts of *C. genistoides* with the use of the HaCaT cell line. Cell viability was conducted using MTT assays. *In vitro* wound healing was tested on HaCaT cells using an established scratch assay method. The effect of *A. muthi-muthi* gel material on HaCaT cell migration was also investigated. The extracts of *C. genistoides* did not demonstrate any *in vitro* wound healing effects. *A. muthi-muthi* gel material exhibited significantly ($p < 0.05$) higher percentage wound closure, compared to the control at all three concentrations investigated.

**Keywords:** Wound healing, *Aloe vera, Aloe ferox, Aloe muthi-muthi, Cyclopia*, honeybush, scratch assay

## Introduction

A wide variety of products, substances and dosage forms are used in the treatment of wounds such as alginates, antimicrobials, foams and hydrocolloids, amongst others (Hess, 2012). Phytochemicals that are found in traditional wound healing remedies commonly have anti-

oxidant or anti-inflammatory properties (Shah & Amini-Nik, 2017). Plants of the *Terminalia* genus are examples of such remedies used in Asian traditional wound healing remedies (Shah & Amini-Nik, 2017). Other plant species that have been investigated for wound healing include *Anagallis arvensis* L. and *A. foemina* Mill., which have shown anti-inflammatory and bacteriostatic properties *in vitro* (López *et al.*, 2011). *Bulbine frutescens* and *B. natalensis* exhibited significant improvement in wound contraction compared to an untreated control in *in vivo* animal studies (Pather *et al.*, 2011). The use of aloe plant material as a wound healing remedy is especially notable, which has already been described in the first century A.D. by Dioscorides (Govaerts & Newton, 2018b; Steenkamp & Stewart, 2007). The wound healing effects of materials from different *Aloe* species have been investigated scientifically with both *in vitro* and *in vivo* experiments. Increased wound area reduction was found in mice treated with *Aloe vera* powder, in combination with poly(lactic-co-glycolic acid) (PLGA) nanofiber dressings and recombinant human epidermal growth factor (rhEGF), in comparison with controls of PLGA and rhEGF only (Garcia-Orue *et al.*, 2016). *Aloe vera, A. ferox* and *A. marlothii* leaf materials (i.e. gel and whole leaf extracts) have demonstrated increased *in vitro* wound healing in a zone exclusion type assay using human immortalised keratinocytes (HaCaT cells) (Fox *et al.*, 2017). The *in vitro* wound healing potential of *A. vera* has also been demonstrated using human primary epidermal keratinocytes (HPEK) and a human skin equivalent model, with increased expression of integrin receptors (β1, α6 and β4) and E-cadherin being observed in HPEK treated with *A. vera* (Moriyama *et al.,* 2016). *Aloe muthi-muthi* has recently been cultivated by means of forced cross-pollination between *A. vera* and *A. ferox* at Rooiklip nursery in Swellendam, South Africa. It has thorny leaves that resemble those of *A. vera*, but also features erect racemes of yellow flowers. The medicinal properties such as the wound healing potential of *A. muthi-muthi* are still unknown.

*Cyclopia* species form part of the Fabaceae plant family (legumes) and are commonly known as honeybush. Honeybush is endemic to the fynbos shrubland in the Western and Eastern Cape provinces of South Africa (Kokotkiewicz & Luczkiewicz, 2009; Joubert *et al.,* 2008). Extracts of these plants are commonly consumed as herbal teas. Honeybush infusions contain a variety of bioactive phytochemicals including benzophenones, xanthones and flavones such as hesperidin (Kamara *et al.*, 2003; Schulze *et al.*, 2015). Herbal teas such as *Athrixia phylicoides* (bush tea) has been traditionally used as a treatment for boils, acne and infected wounds (Joubert *et al.*, 2008). Extracts of *Cyclopia* species have shown some health effects such as tumour inhibition, which has been attributed to flavonols, flavones, proanthocyanidin, as well as other phytochemicals present in these extracts (Marnewick *et al.*, 2005). Since the wound healing potential of *Cyclopia* species is unknown, it was decided to also include extracts of honeybush in this *in vitro* wound healing study.

## Results and Discussion

### Characterisation of *Aloe muthi-muthi* gel and whole leaf material

The $^{1}$H-NMR spectra for the gel and whole leaf materials are shown in Figure 1a and 1b, respectively. The content of marker molecules in the *A. muthi-muthi* gel and whole leaf materials that were obtained from quantitative $^{1}$H-NMR are shown in Table 1.

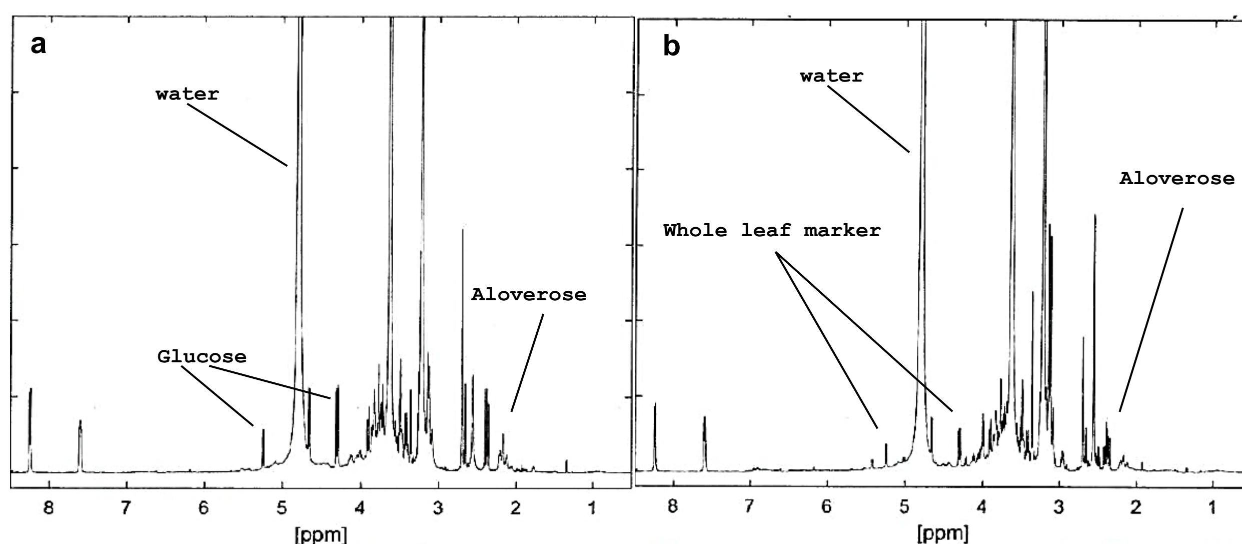


**Figure 1:** **$^{1}$H-NMR spectra for *A. muthi-muthi* gel (a) and *A. muthi-muthi* whole leaf (b) material**

**Table 1:** **Quantity of marker molecules in *Aloe muthi-muthi* gel and whole leaf materials determined by $^{1}$H-NMR spectroscopy**

| Component | *Aloe muthi-muthi* gel | | *Aloe muthi-muthi* whole leaf | |
|---|---|---|---|---|
| | Content (%) | Content (mg/ml) | Content (%) | Content (mg/ml) |
| Aloverose (polysaccharide) | 11.3 | 793.8 | 8.1 | 568.3 |
| Glucose | 11.7 | 821.1 | 6.8 | 477.2 |
| Malic acid | 10.4 | 730.8 | 5.4 | 380.3 |
| Lactic acid | 0.1 | 12.5 | Traces | |
| Citric acid | Not detected | | 1.5 | 103.8 |
| Isocitric acid (whole leaf marker) | Not detected | | 5.1 | 355.8 |

As expected, the composition as listed in Table 1 and Figure 1 for *A. muthi-muthi* is similar to that of *A. vera* and *A. ferox* with respect to the type and quantity of marker chemical compounds (Fox *et al.,* 2017).

**Characterisation of *Cyclopia genistoides* extracts**

The quantity of specific benzophenone and xanthone marker molecules present in both the honeybush crude extracts and enriched fractions, as determined by high performance liquid chromatography (HPLC), is shown in Table 2. The specific benzophenone marker molecules quantified included 3-β-D-glucopyranosyl iriflophenone (I3G), 3-β-D-glucopyranosyl-4-β-D-glucopyranosyl oxyiriflophenone (IDG) and 3-β-D-glucopyranosyl maclurin (M3G), while the specific xanthone marker molecules were mangiferin and isomangiferin.

**Table 2: Quantities of specific benzophenones and xanthones determined by HPLC in the honeybush crude extracts and fractions**

| Extract/fraction | (g/100 g) | | | | |
|---|---|---|---|---|---|
| | Benzophenones | | | Xanthones | |
| | I3G | IDG | M3G | Mangiferin | Isomangiferin |
| **ARC 188** | 3.885 | 2.214 | 1.101 | 13.811 | 3.193 |
| **ARC 188 fX** | 5.804 | 0.000 | 0.000 | 54.016 | 14.352 |
| **ARC 188 Benz** | 28.272 | 23.267 | 11.384 | 0.000 | 0.000 |
| **ARC 2013** | 4.298 | 1.935 | 1.076 | 12.812 | 3.229 |

**Methyl thiazolyl tetrazolium (MTT) assays**

MTT assay results for ARC 188 and ARC 2013 crude extracts are shown in Figure 2 and the results for the enriched fractions are displayed in Figure 3. The MTT assay results for *Aloe muthi-muthi* gel and whole leaf material are shown in Figure 4.

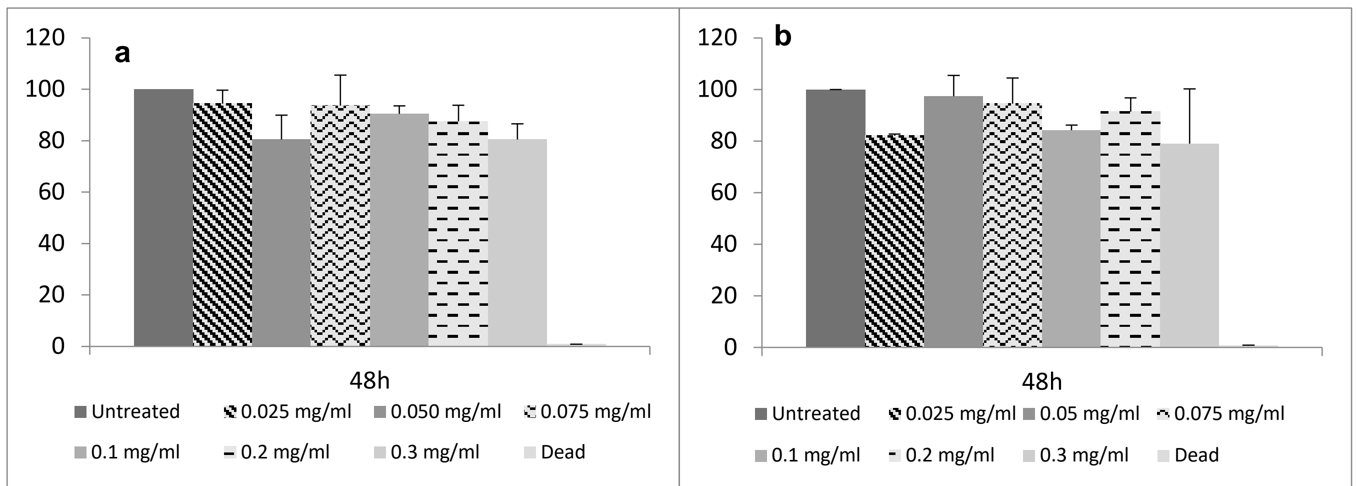


**Figure 2:** **Percentage viability of HaCaT cells (MTT assay) after 48 h exposure to honeybush crude extracts: (a) ARC 188 and (b) ARC 2013**

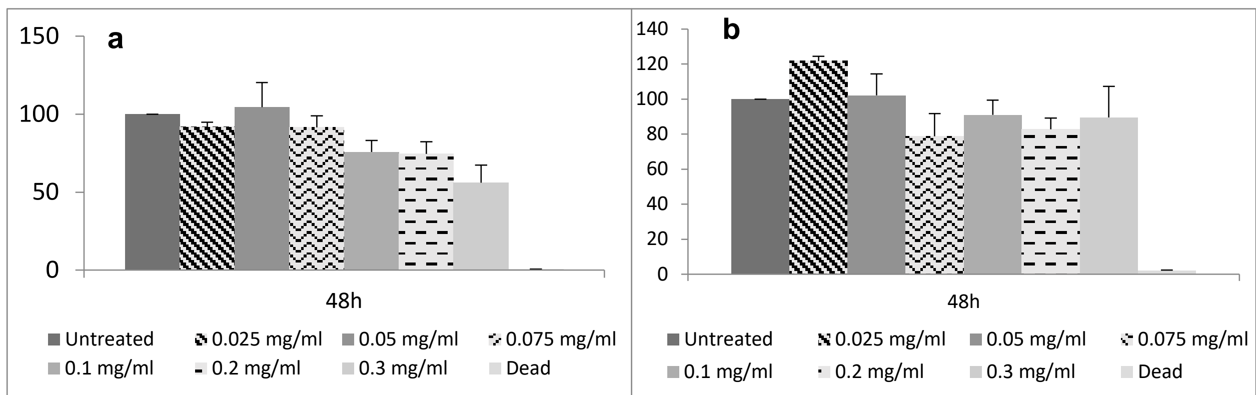


**Figure 3:** **Percentage viability of HaCaT cells (MTT assay) after 48 h exposure to (a) ARC 188 Benz, (b) ARC 188 fX fractional extracts**

It is evident from the data depicted in Figure 2 and 3 that after an exposure period of 48 h to the honeybush extracts, some reduction in cell viability occurred as indicated by MTT-assay (i.e. reduction in mitochondrial metabolism), indicating a moderate level of cytotoxicity at higher concentrations (López-García *et al.,* 2014). However, none of the extracts of *C. genistoides* demonstrated cell death in the HaCaT cell model at any of the experimental concentrations. This indicated the honeybush extracts are not cytotoxic to keratinocyte cells at appropriate concentrations and should be safe for use on the skin.

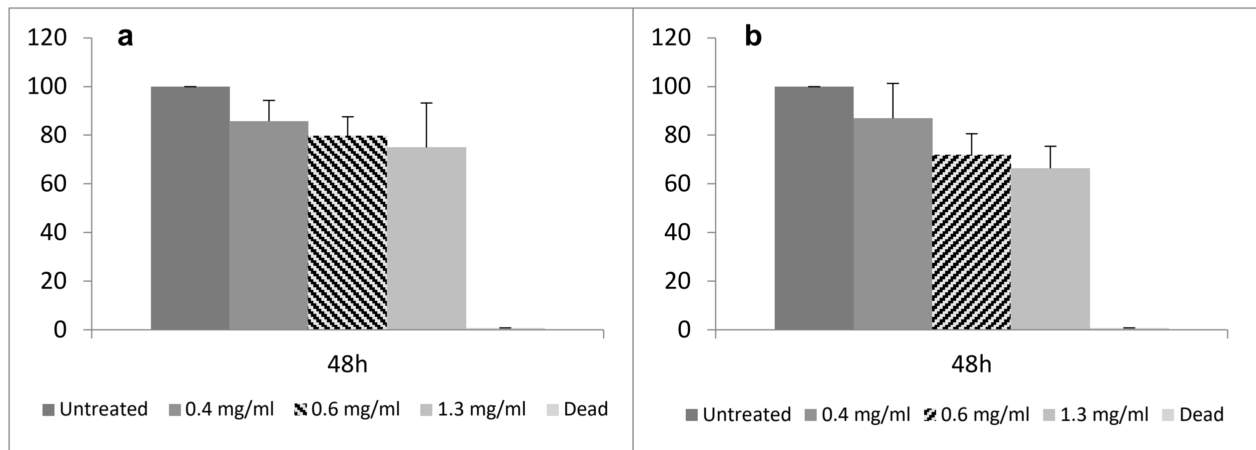


**Figure 4: Viability of HaCaT cells after 48 h exposure to *A. muthi-muthi* gel (a) and *A. muthi-muthi* whole leaf (b) plant material**

The *A. muthi-muthi* gel and whole leaf materials showed a concentration dependent decrease in cell viability as measured with MTT-assay (i.e. reduction in mitochondrial metabolism), but did not indicate any death of the HaCaT cells. This is in line with previous findings of the gel and whole leaf materials of different aloe species (Du Plessis and Hamman, 2014; Fox *et al.,* 2017).

**Scratch assay**

None of the honeybush crude extracts or fractions investigated in this study (ARC 2013, ARC 188, ARC 188 Benz, ARC 188 fX, ARC 2013) resulted in improved wound gap closure percentages compared to the untreated control, as shown in Figure 5. In congruence with the percentage wound closure results, none of the *C. genistoides* extracts and fractions showed wound closure rates higher than the untreated control. In Figure 6, images of wound gaps in HaCaT cells exposed to the highest concentration (0.3 mg/ml) of ARC 188 at different time points are displayed together with wound gaps in untreated HaCaT cells at the same time points. These images clearly show that almost no difference could be observed between the cells treated with the ARC 188 honeybush extract and the untreated control.

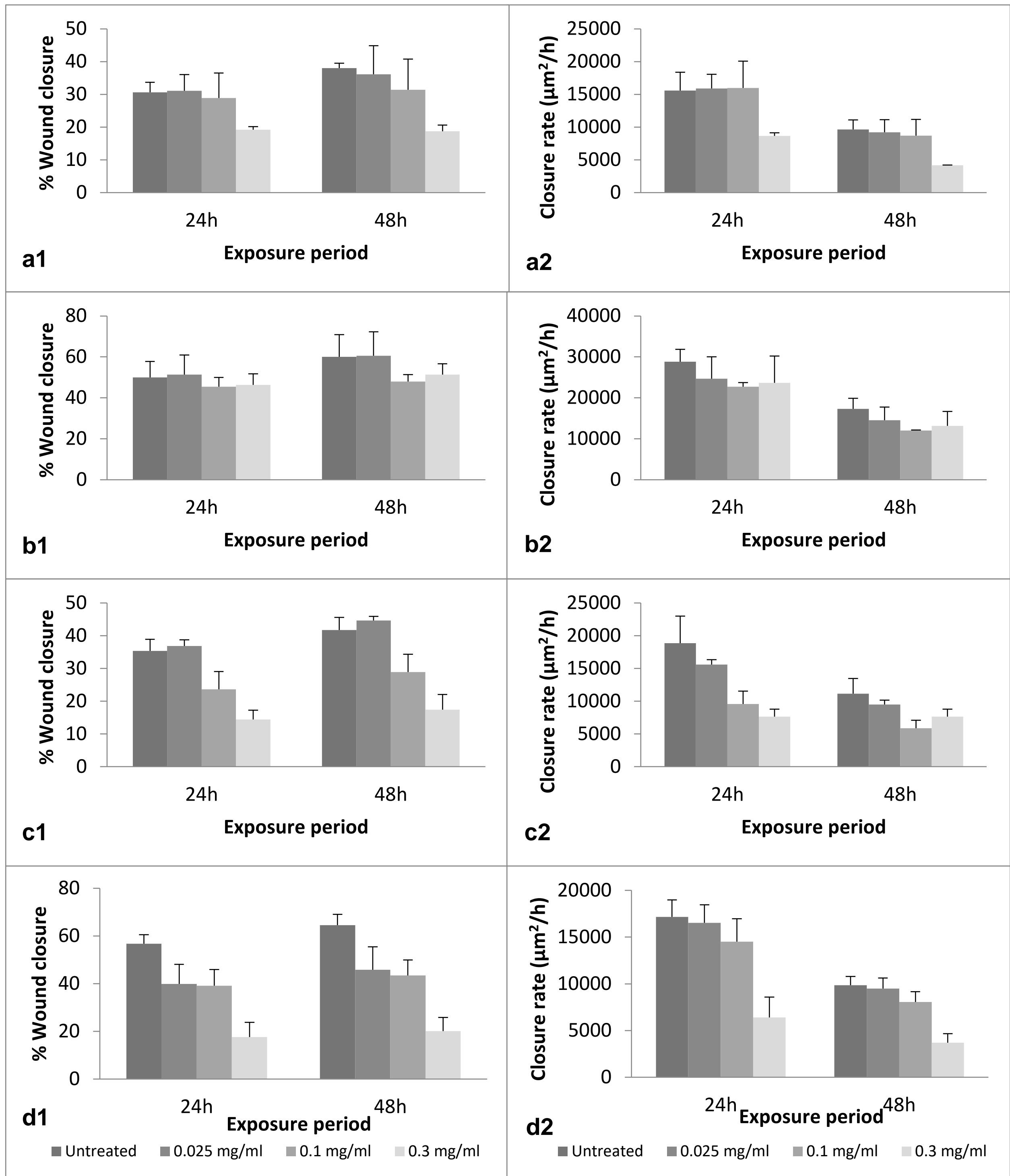


**Figure 5:** **Wound closure (1) and wound closure rate (2) results after exposure to (a) ARC 188, (b) ARC 188 Benz, (c) ARC 188 fX and (d) ARC 2013 at 24 h and 48 h treatment periods**

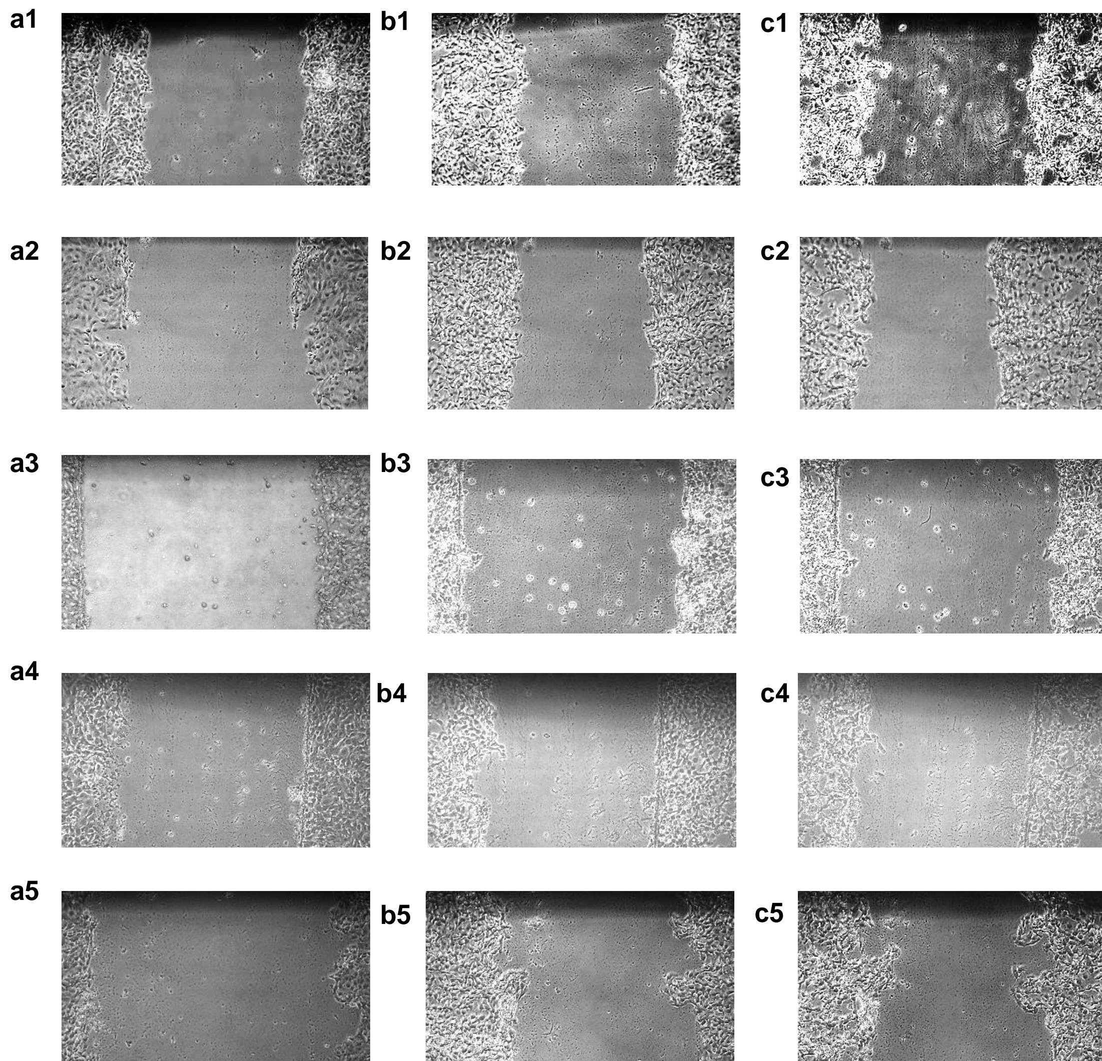


**Figure 6: Microscopic photos of wound gaps in HaCaT cells introduced by the scratch technique: 1) after treatment with ARC 188 at 0.3 mg/ml (a1 at 0 h, b1 at 24 h and c1 at 48 h), 2) ARC 188 Benz at 0.3 mg/ml (a2 at 0 h, b2 at 24 h and c2 at 48 h), 3) ARC 188 fX (a3 at 0 h, b3 at 24 h and c3 at 48 h), 4) ARC 2013 (a4 at 0 h, b4 at 24 h and c4 at 48 h) and 5) an untreated control (a5 at 0 h, b5 at 24 h and b5 at 48 h)**

The percentage wound closure results for *A. muthi-muthi* gel and whole leaf material are presented in Figure 7. Microscopic images depicting the wound gap closure results after 24 and 48 h of treatment with *A. muthi-muthi* gel and whole leaf material are depicted in Figure 8 and Figure 9 for the gel and whole leaf, respectively. It is evident from Figure 8 that the application of *A. muthi-muthi* gel to HaCaT cells with scratched wound gaps resulted in notable improvement in wound gap closure in comparison to the untreated control. The percentage wound closure results as depicted in Figure 7 indicated the effectiveness of *A. muthi-muthi* gel with respect to wound healing, exhibiting 2.0-fold to 2.5-fold higher wound closure percentages

in comparison to the untreated control. This notable improvement in wound closure, which was statistically significant (ANOVA, $p < 0.05$) was obtained at all three concentrations of *A. muthi-muthi* gel investigated in this study. The improvement in wound healing for the *A. muthi-muthi* gel material was concentration dependent with the highest concentration of 1.3 mg/ml resulting in the highest percentage wound closure. In accordance with the percentage wound closure caused by the *A. muthi-muthi* gel material, an increase in the closure rate (Figure 7) of the wounds were also found in comparison to the untreated control, but only the wound closure rates of the 0.6 and 1.3 mg/ml concentrations were statistically significant (ANOVA, $p < 0.05$).

In contrast to the promising wound healing results obtained with the *A. muthi-muthi* gel, the *A. muthi-muthi* whole leaf material (Figure 9) did not give similar wound healing results. Both wound gap closure and closure rate were not increased in comparison to the untreated control. This can possibly be attributed to differences in the chemical composition of the whole leaf material in comparison to the gel material. As shown in Table 1, the whole leaf material contained less aloverose (a bioactive polysaccharide in aloe gel material) than the gel material, but more citric acid and isocitric acid. These chemical differences are due to the inclusion of leaf rind material in the *A. muthi-muthi* whole leaf material that was not part of the *A. muthi-muthi* gel material.

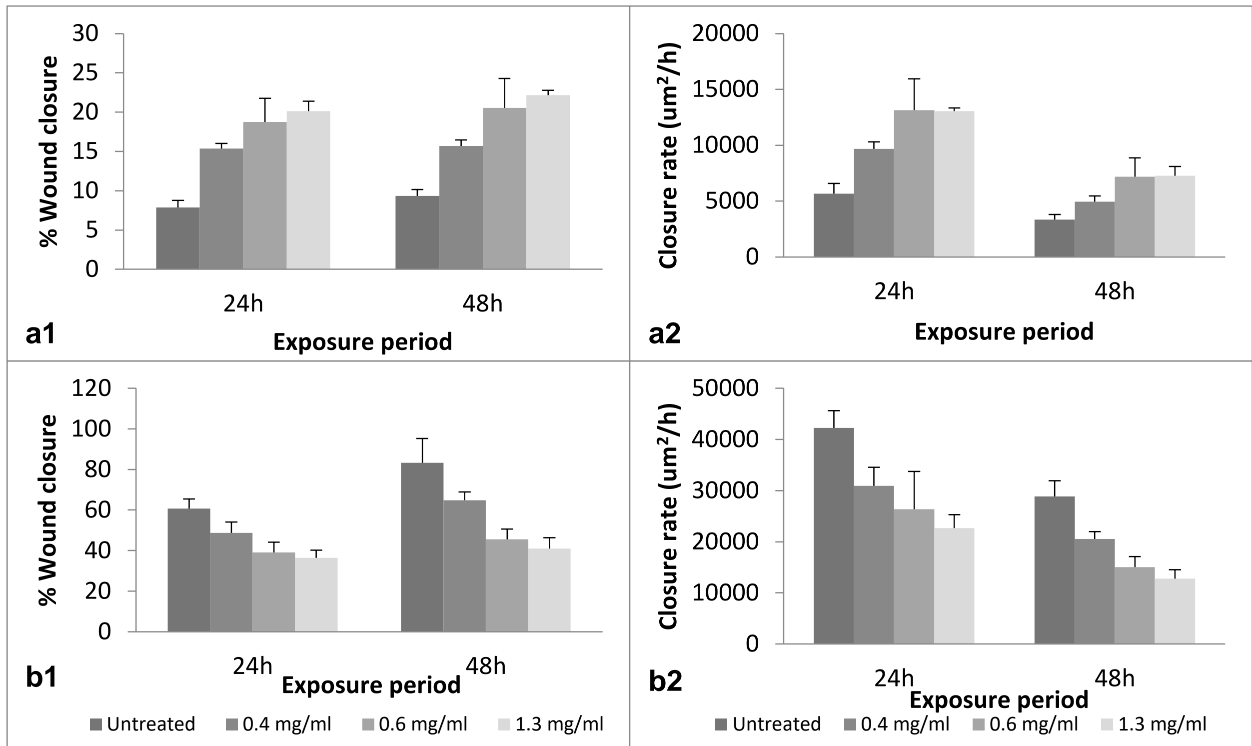


**Figure 7:** **Wound closure (1) and wound closure rate (2) results after exposure to (a) *A. muthi-muthi* gel and (b) *A. muthi-muthi* whole leaf at 24 h and 48 h treatment periods**

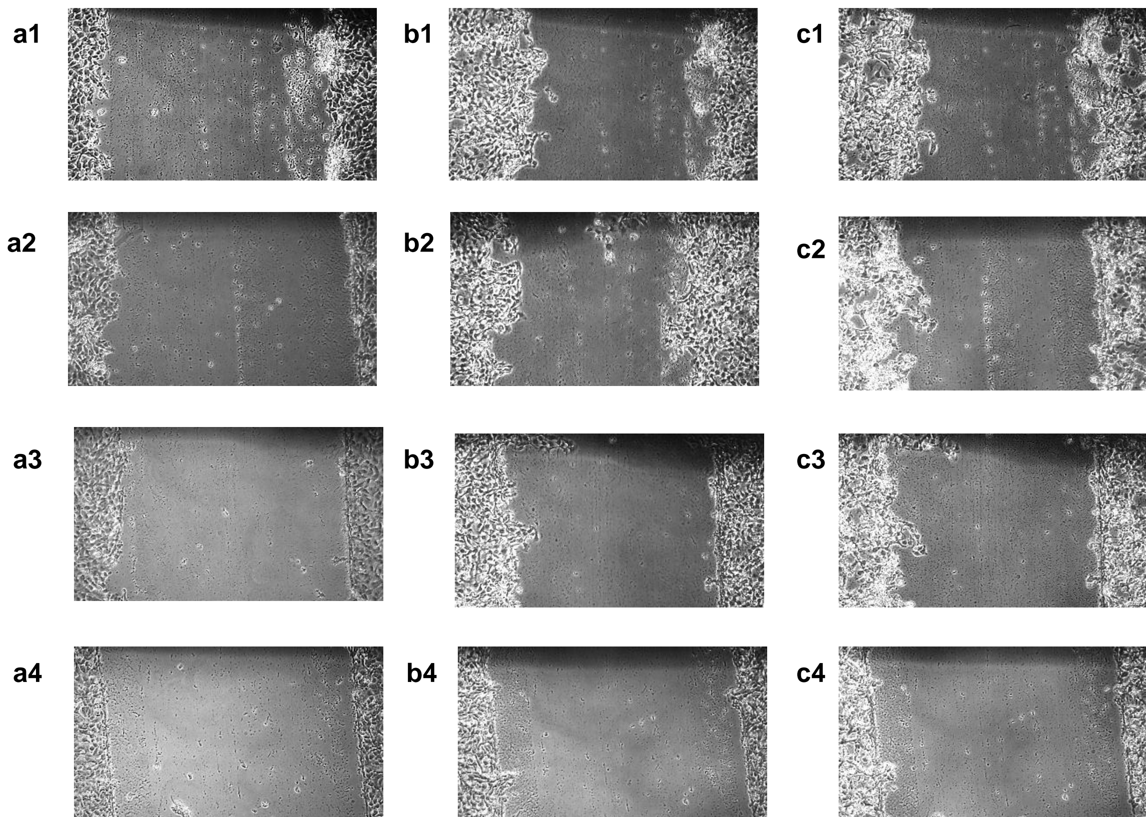


**Figure 8:** **Microscopic photos of wound gaps in HaCaT cells introduced by the scratch technique after treatment with *A. muthi-muthi* gel at 1.3 mg/ml (a1 at 0 h, b1 at 24 h and c1 at 48 h), 0.6 mg/ml (a2 at 0 h, b2 at 24 h and c2 at 48 h) and 0.4 mg/ml (a3 at 0 h, b3 at 24 h and c3 at 48 h) compared to an untreated control (a4 at 0 h, b4 at 24 h and c4 at 48 h)**

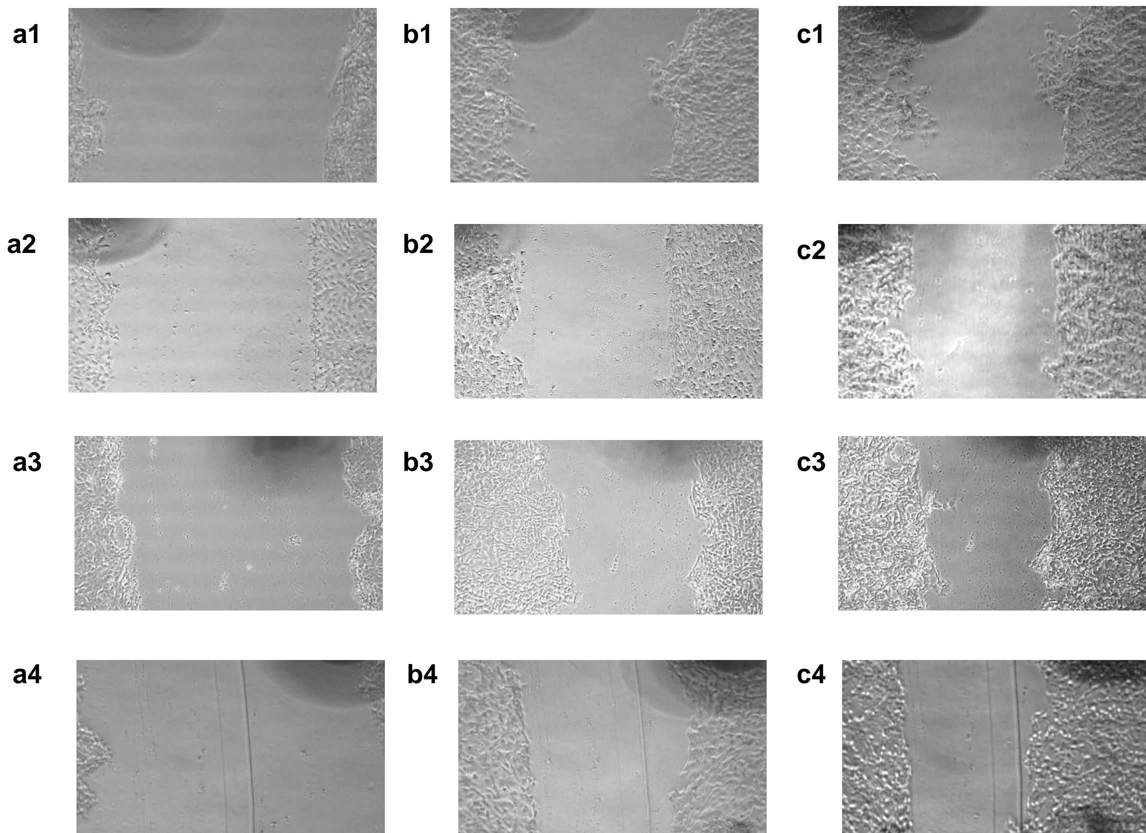


**Figure 9: Microscopic photos of wound gaps in HaCaT cells introduced by the scratch technique after treatment with *A. muthi-muthi* whole leaf material at 1.3 mg/ml (a1 at 0 h, b1 at 24 h and c1 at 48 h), 0.6 mg/ml (a2 at 0 h, b2 at 24 h and c2 at 48 h) and 0.4 mg/ml (a3 at 0 h, b3 at 24 h and c3 at 48 h) compared to an untreated control (a4 at 0 h, b4 at 24 h and c4 at 48 h)**

***In vitro* cell migration assay**

The migration results after exposure of HaCaT cells for 24 h to the different concentrations of *A. muthi-muthi* gel are depicted in Figure 10. In general, the lowest concentration (0.4 mg/ml) of the *A. muthi-muthi* gel resulted in a decrease in cell migration compared to the untreated control, while a concentration dependent improvement in cell migration was observed for the 0.6 and 1.3 mg/ml concentrations of the *A. muthi-muthi* gel material. The increase in cell migration in comparison to the untreated control was, however, not statistically significant ($p > 0.05$). This showed that cell migration may play a role in wound healing at higher concentrations of *A. muthi-muthi* gel.

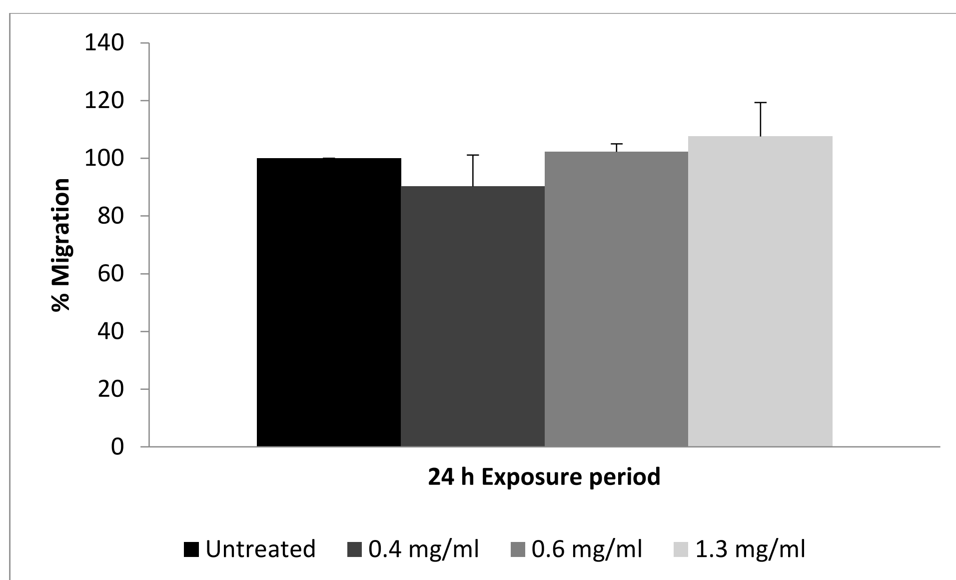


**Figure 10: Cell migration results of HaCaT cells treated with *A. muthi-muthi* gel compared to an untreated control**

## Materials and Methods

### Preparation of *Aloe muthi-muthi* whole leaf and gel material

Leaves of *Aloe muthi-muthi* plants were provided by Mr. Jaap and Hannes Viljoen of Rooiklip nursery in Swellendam, South Africa. The inner gel material and outer rind were separated by manually filleting the leaves with knives. The gel material was liquidised in a kitchen blender and the outer rind parts were pulverised using a Retsch MM400 mixer mill (Retsch GmbH). Powdered materials were obtained by freeze drying of the gel material and the whole leaf separately. To prepare *A. muthi-muthi* whole leaf material, a quantity of the rind was added to the gel material in a ratio that reflects the approximate ratio of gel:rind of a real whole *A. muthi-muthi* leaf.

### Characterisation of *Aloe muthi-muthi* gel and whole leaf materials

The *Aloe muthi-muthi* gel and whole leaf plant material were chemically characterised with quantitative proton nuclear magnetic resonance spectroscopy ($^1$H-NMR) and spectra were obtained from a Bruker Avance III HD NMR. The quantities of marker molecules (i.e. aloverose, glucose, malic acid, lactic acid, citric acid and whole leaf marker) in the *A. muthi-muthi* gel and whole leaf materials were determined according to a previously published method (Jiao *et al.*, 2010).

**Collection and preparation of *C. genistoides* (honeybush) extracts**

The unfermented shoots of the plant material supplied by the *Cyclopia* gene bank of the ARC at Nietvoorbij, Stellenbosch were dried to 10% moisture by using a cross-flow drying tunnel set to 40°C for 16 h. For crude extract ARC 188, thick shoots were separated and only leaves and fine stems were used.

For the ARC 188 crude extract, a water-ethanol (40% v/v) solvent mixture was prepared in a 5.0 l volumetric flask. The 99% v/v ethanol used for this preparation was supplied by Servochem (Pty) Ltd. In 2.0 l glass bottles, 1.5 l of solvent was added to the plant material and the bottles closed tightly. The samples were then placed at 93°C in a water bath for 30 min, during which the samples were mixed periodically every 5 min. After being poured through a 200 mesh stainless steel sieve, the extractions were filtered using Whatman #4 filter paper. The solvent was evaporated from the extraction solutions using a Büchi Rotavapor R-125 (BÜCHI Labortechnik). Enriched fractions (including ARC 188 fX, ARC 188 Benz) were prepared from the ARC 188 crude extract by performing mini-scale extractions. These extractions were then characterised and pooled according to their benzophenone and xanthone content using XAD fractionation with the use of XAD1180 polymeric resin (Table 3).

A crude aqueous extract of *C. genistoides* (ARC 2013) was also prepared on the plant material sourced from the gene bank of the ARC. Extraction was done using the same process as the ARC 188 crude extract with the exception that only water was used, instead of an ethanol/water mixture for the extraction process.

**Table 3: List of *Cyclopia genistoides* crude extracts and enriched fractions prepared for this study**

| Crude extract/fraction | Abbreviation | Marker molecules present |
|---|---|---|
| ARC 188 crude ethanolic extract | ARC 188 | IDG, M3G & I3G (Benzophenones)<br>Mangiferin & isomangiferin (xanthones) |
| ARC 188 Xanthone fraction | ARC 188 fX | I3G (Benzophenone)<br>Mangiferin & isomangiferin (xanthones) |
| ARC 188 Benzophenone fraction | ARC 188 Benz | IDG, M3G and I3G (Benzophenones) |
| ARC 2013 aqueous extract | ARC 2013 | IDG, M3G & I3G (Benzophenones)<br>Mangiferin & isomangiferin (xanthones) |

**Characterisation of *C. genistoides* extracts**

High performance liquid chromatography with diode-array detection (HPLC-DAD) was used to characterise the *C. genistoides* extracts based on the method described by Beelders *et al.*

(2014). An Agilent 1200 series system was used. To prepare the column, approximately 1.2 kg of XAD1180 resin was prepared in 100% methanol (Merck Millipore) on the day prior to characterisation. An open glass column was filled with the XAD1180 resin and column dimensions were 70 mm for the internal diameter and 550 mm for the column height. Spectra were recorded between 200 and 700 nm. Selective wavelength monitoring was done at 288 nm for the benzophenones IDG and I3G and at 320 nm for the xanthones mangiferin and isomangiferin as well as the benzophenone maclurin.

**Culturing of HaCaT cells**

HaCaT cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (HyClone Laboratories Inc.), supplemented with 10% foetal bovine serum (FBS) (Thermo Fisher Scientific LLC), 1% penicillin/streptomycin (Lonza AG, 10000 U/ml penicillin and 10000 U/ml streptomycin), 1% non-essential amino acids (NEAA) (Lonza AG) and 2 mM L-glutamine (Lonza AG). The cells were maintained in a humidified atmosphere at 37°C and 5% $CO_2$ using an ESCO CelCulture® $CO_2$ incubator (ESCO technologies Inc.). Cells were cultured in T75 $cm^2$ flasks (Corning® Costar® Corporation). Growth medium was changed every 48 to 72 h and cells were viewed under a Nikon TS100 light microscope (Nikon Instruments) in order to estimate confluence.

**Sub-culturing of HaCaT cells**

HaCaT cells were sub-cultured by trypsinisation at 70% to 80% confluency. Spent growth medium was removed from the culture flasks and the cells were rinsed twice with 10 ml phosphate buffered saline (PBS) (Thermo Fisher Scientific LLC) to remove any residual medium. After the addition of 3 ml trypsin EDTA (Lonza AG), the flasks were incubated for 12 min at 37°C. To neutralise the trypsin, 6 ml preheated growth medium was added to the flasks and washed thoroughly to remove all the cells from the flask surface. The cell suspensions were centrifuged at 140 *x g* for 5 min and the supernatant removed without disturbing the cell pellets. The pellets were then resuspended in growth medium and the suspension divided to a 1:10 to 1:15 ratio into new flasks. Preheated growth medium was then added to the flasks to a final volume of 15 ml and the flasks returned to the incubator.

**Methyl thiazolyl tetrazolium (MTT) cell viability assay**

Viability was measured after 48 h with an MTT-assay to evaluate the cytotoxicity of the plant materials at tested concentrations. HaCaT cells were seeded in 96-well plates (Corning® Costar® Corporation) at 100000 cells/ml by pipetting 200 µl into each well and incubating for 24 h. The experimental groups consisted of six concentrations for honeybush extracts (0.025,

0.050, 0.075, 0.100, 0.200 and 0.300 mg/ml) and three concentrations for *Aloe muthi-muthi* (0.4, 0.6 and 1.3 mg/ml). In addition to the experimental groups, both a dead cell control (treated with 0.2% Triton X-100) and an untreated control were tested, as well as a dimethyl sulfoxide (DMSO) blank group without any cells. All experimental and control groups were tested in triplicate. After the 24 h incubation, the medium of the experimental and control groups was removed and replaced with medium containing plant material added at the relevant experimental concentrations. The experimental concentrations were prepared by preparing stock solutions with DMEM and then further diluting the stock solutions to the relevant experimental concentrations. Prior to dilution, the stock solutions were filtered using a 0.45 µm syringe filter. Control groups received medium without added plant material. The plates were then incubated.

At time point 48 h, after experimental solutions were added to the HaCaT cells, the medium in all experimental groups and the untreated control group was aspirated and the cells washed twice with 100 µl PBS. The dead cell control group was treated with Triton X-100 (Sigma-Aldrich Corporation) and the Triton X-100 was removed after 15 min. A volume of 180 µl serum- and additive-free DMEM along with 20 µl MTT solution (Sigma-Aldrich Corporation) (5 mg/ml stock solution in PBS) was added to the experimental and control groups. The plates were then covered with aluminium foil and incubated for 90 min. After the incubation period, the MTT medium was removed and 200 µl DMSO (Sigma-Aldrich Corporation) were added to every experimental group, control group and the DMSO blank group, in order to dissolve the formazan crystals that formed during incubation. The plate was consequently placed on an orbital shaker for 1 h to dissolve the formazan completely. After 1 h, the absorbance was measured at 560 nm and 630 nm, respectively.

Cell viability was consequently calculated using the following equation:

$$\% \text{ cell viability} = (\Delta\text{Sample} - \Delta\text{Blank}) / (\Delta\text{Control} - \Delta\text{Blank}) \times 100$$

$\Delta$Sample = Absorbance of treated cells$_{560}$ – Absorbance of treated cells$_{630}$
$\Delta$Blank = Absorbance of DMSO blank$_{560}$ – Absorbance of DMSO blank$_{630}$
$\Delta$Control = Absorbance of untreated control$_{560}$ – Absorbance of untreated control$_{630}$

### ***In vitro* wound healing scratch assay**

HaCaT cells were trypsinised at 80% confluence and counted with trypan blue (Sigma-Aldrich Corporation) using a haemocytometer (Marienfeld Superior™, 0.0025 mm$^2$). A cell suspension of 400000 cells per ml was prepared. A volume of 2.5 ml of this prepared cell suspension was seeded into each well of a 12-well plate (Corning® Costar® Corporation). The plate was then

incubated at 37°C in a 5% $CO_2$ humidified atmosphere for 24 h. After 24 h, the cells were visualised under a microscope to ensure the formation of a monolayer in every well. Experimental solutions of the selected plant materials were prepared by preparing a stock solution of plant material in DMEM growth medium and diluting to appropriate concentrations (0.025, 0.100 and 0.300 mg/ml for *C. genistoides* and 0.400, 0.600 and 1.300 mg/ml for *A. muthi-muthi*). Both *A. muthi-muthi* gel and whole leaf materials, along with ARC 188 and ARC 2013 crude extracts, as well as ARC 188 fX and ARC 188 Benz fractions were tested for wound healing. Scratches were induced in the monolayers across the diameter of the wells using a 200 µl pipet-tip (Liang *et al.,* 2007). The culture medium in each well was aspirated and the wells washed 4 times with serum- and additive-free DMEM. A volume of 4 ml of each experimental concentration (culture medium for untreated control) was added to the wells in triplicate. Photos of each well were taken immediately after scratches were induced (0 h) and at 8 h intervals thereafter, for a total period of 48 h. The photos were taken with a camera (The Imaging Source DFK 72AUC02) mounted to a Nikon TS100 light microscope (Nikon instruments). The surface areas of the scratches were measured using ImageJ software (National institutes of health, USA). The software was calibrated using a bitmap image format calibration photo for 10 x magnification. For the duration of the experiment, the plates were incubated in a 95% humidified and 5% $CO_2$ environment at 37°C.

***In vitro* cell migration assay**

The effects of *A. muthi-muthi* gel (selected based on the wound closure results) on cell migration was evaluated using the Cell Biolabs CytoSelect™ 24-Well Cell Migration and Invasion Assay kit (Cell Biolabs, Inc.). The kit consisted of a 24-well cell culture plate with 12 polycarbonate membrane inserts (8 µm pore size). Cell suspensions containing 1 x $10^6$ cells/ml were prepared, having been resuspended in serum and additive-free DMEM after sub-culturing. The suspensions were centrifuged at 140 *x g* for 5 min. Each individual pellet was then resuspended with 1 ml serum- and additive-free DMEM containing the same experimental concentrations of *A. muthi-muthi* gel used in the scratch assay (0.4, 0.6 and 1.3 mg/ml). The untreated control was resuspended in serum and additive-free DMEM. A volume of 500 µl of culture medium (with additives) was added to each well (outside the inserts). A volume of 300 µl of each prepared suspension containing the experimental concentrations, as well as 300 µl of the untreated control were added to the inside of the inserts. The migration was seeded in triplicate. The plates were then incubated in 95% humidified and 5% $CO_2$ air for 24 h. After the incubation period, the media in each insert was aspirated and wetted cotton-tipped swabs were used to gently clean out the interior of each well. Each insert was transferred to a clean well containing 400 µl of the supplied stain solution and incubated for 10 min at room temperature.

The inserts were then gently rinsed using a beaker of water and allowed to air dry. Each insert was transferred to another clean well, containing 200 µl of the supplied extraction solution and incubated for 10 min on an orbital shaker. A volume of 100 µl of each sample was added to a 96-well microtiter plate and the absorbance measured at 560 nm.

#### Statistical analysis

The percentage wound gap closure data and cell migration data were analysed for statistically significant differences with STATISTICA Ver 12. Tukey's post-hoc tests were performed. Differences were accepted when $p < 0.05$.

### Conclusion

*Aloe muthi-muthi* gel and whole leaf materials, as well as different extracts (ethanolic and aqueous crude extracts, xanthone and benzophenone rich fractions) of *Cyclopia genistoides* were investigated for wound healing properties, using the scratch assay on the HaCaT cell culture model. MTT-assays indicated that none of the plant materials at the concentration ranges investigated in this study showed cytotoxic effects on HaCaT cells. Unfortunately, none of the crude extracts or fractions of *C. genistoides* exhibited any *in vitro* wound healing characteristics. On the other hand, *A. muthi-muthi* gel exhibited significant wound healing properties as indicated by a statistically significant increase in percentage wound gap closure and migration rate, for the highest two concentrations used in this study in comparison to an untreated control. *Aloe muthi-muthi* whole leaf material showed some wound healing effects, but to a much lower extent than the gel material and it was not statistically significant.


### Acknowledgements

The authors would like to thank Rooiklip nursery in Swellendam, South Africa for the provision of the *A. muthi-muthi* leaves.


### Conflict of interest statement

The authors declare no conflict of interest.

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# CHAPTER 4 CONCLUSION AND FUTURE PROSPECTS

## 4.1 Conclusion

*Aloe vera* and *A. ferox* have a history of traditional use as wound healing remedies (Hamman, 2008; Steenkamp & Stewart, 2007). The wound healing potential of these species has also been reported in literature (Fox *et al.*, 2017; Jia *et al.*, 2008). *Cyclopia* species, commonly called honeybush, have been found to be a source of various phytochemicals which are biologically active (Joubert *et al.*, 2008; Kokotkiewicz & Luczkiewicz, 2009). The wound healing potential of these plants is mostly unknown, warranting investigation into the *in vitro* wound healing effects of these plants. Re-epithelialisation has been proposed as the primary mechanism of skin wound healing in humans, other than in loose skinned mammals where skin wounds heal primarily by wound contraction (Sullivan *et al.*, 2001). Therefore, for an *in vitro* wound healing assay, HaCaT cells serve as a suitable model as these cells can be used as an *in vitro* analogue for human epidermis (Lehmann, 1997).

Firstly, the aim of this study was to evaluate whether *in vitro* wound healing effects corresponding with *A. vera* and *A. ferox* as previously published, could be observed with *A. muthi-muthi* gel and whole leaf materials. Secondly, the *in vitro* wound healing effects of *C. genistoides* extracts were evaluated.

The cytotoxicity of both the *A. muthi-muthi* plant materials and the *C. genistoides* extracts were tested with an MTT-assay in the HaCaT cell model after an exposure period of 48 h. A range of experimental concentrations were selected for the cytotoxicity studies including 0.025, 0.050, 0.075, 0.100, 0.200 and 0.300 mg/ml. The MTT assay results, which serve as an indication of the cells' ability to metabolise MTT to its insoluble formazan form, showed that no severe cytotoxic effects occurred in HaCaT cells exposed to the *C. genistoides* extracts, even at the highest concentrations investigated. For *A. muthi-muthi,* three concentrations that were used in a previous *in vitro* wound healing experiment were also selected for this study which included 0.4, 0.6 and 1.3 mg/ml. The HaCaT cells exposed to *A. muthi-muthi* gel and whole leaf plant material exhibited a concentration dependent increase in cytotoxicity compared to an untreated control. However, the *A. muthi-muthi* plant materials did not show such severe cytotoxicity as to induce cell death.

Wound healing was simulated *in vitro* by means of a scratch assay, where a pipet tip was used to induce a wound gap in a monolayer of HaCaT cells by scratching across the diameter of the cell monolayers in the wells (Liang *et* al., 2007). For the *C. genistoides* extracts, three concentrations were selected (i.e. 0.025, 0.100 and 0.300 mg/ml) for the wound healing scratch

assay. The same *A. muthi-muthi* concentrations were used in the scratch assay as in the MTT cytotoxicity experiments. None of the extracts of *C. genistoides* exhibited any notable improvement in *in vitro* wound closure when compared to an untreated control. Corresponding with what has been demonstrated with *A. vera* and *A. ferox* in a previous study, the hybrid *A. muthi-muthi* gel material did exhibit *in vitro* wound healing effects with significant improvement in the closure and closure rate of induced scratches in monolayers of cultured HaCaT cells when compared to an untreated control (Fox *et al.*, 2017). On the other hand, *A. muthi-muthi* whole leaf material did not show significant improvement in wound closure or closure rate when compared to an untreated control. Furthermore, the effects of *A. muthi-muthi* gel on HaCaT cell migration were tested using a cell migration assay kit using the same concentrations as for the scratch assay (Cell Biolabs, Inc., 2017). The lowest concentration of *A. muthi-muthi* gel (0.4 mg/ml) resulted in a reduction in cell migration, but concentration dependent improvement was observed with increasing concentrations (0.6 and 1.3 mg/ml), although it was not statistically significant ($p > 0.05$).

### 4.2 Future prospects

Based on the results, observations and conclusions of this study, the following future prospects for further investigation are recommended:

- Investigation into the *in vitro* wound healing effects of both the *C. genistoides* extracts and *A. muthi-muthi* plant material with scratch assays and migration assays using fibroblasts as cell model. The reason for this is that fibroblast cells play an important role in terms of cell migration and chemical signalling during the wound healing process.
- Incorporating recombinant human epidermal growth factor (rhEGF) into further investigations into the wound healing effects of *A. muthi-muthi* when using HaCaT cells. This can be used to attempt to simulate an element of the chemical signalling that occurs during the wound healing process.
- To conduct advanced cell culture studies on *A. muthi-muthi* in order to determine the mechanism of wound healing (e.g. cell proliferation).
- To formulate *A. muthi-muthi* gel material into different dosage forms for application to the skin to treat minor wounds (e.g. wound dressings, gel formulations or sprays).

## 4.3 References


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# APPENDIX A – CONFERENCE ABSTRACT AND CERTIFICATE OF ATTENDANCE

**Effects of selected plant materials on *in vitro* wound healing using the HaCat cell culture model**

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Aloe species have been used as a natural remedy for wounds and other ailments for millennia. *Aloe vera* is probably one of the most studied medicinal plants and has demonstrated wound healing and other therapeutic effects in both *in vivo* and *in vitro* studies. Likewise, *Aloe ferox*, endemic to Southern Africa, is used as a health remedy. No research has yet been published on the wound healing effects of *Aloe muthi-muthi*, a hybrid of *A. vera* and *A. ferox*. *Cyclopia* species, colloquially known as honeybush, which are endemic to the fynbos biome of South Africa, are consumed for health promoting reasons due to their antioxidant and polyphenol content. Research on potential health benefits of this genus is plentiful, but there is limited information available on its wound healing potential. Both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red assays were used to establish cell viability after exposure of HaCat cells to extracts of the selected plant materials (i.e. *A. muthi-muthi* and *C. genistoides*) for a period of 48 h. To simulate wound healing *in vitro*, a scratch assay was performed where the closure of the induced scratches in HaCat monolayers was monitored over a 48 h period after application of solutions of the selected plant materials. Migration rates were determined by taking photos of the scratches at 8 h intervals and measuring the surface area of the induced wound. The ethanolic honeybush plant extracts did not show meaningful effects on HaCat cell migration rate, while notable increases in migration rate could be observed in HaCat cells treated with *A. muthi-muthi* gel. These results therefore showed the potential use of *A. muthi-muthi* gel in promoting wound healing.

[274 words]

**This serves to confirm that**

# Morné Fouché

**attended the**

**First Conference of Biomedical and Natural Sciences and Therapeutics (CoBNeST) 2018**

**at**

**Spier Conference Centre, Stellenbosch, and Cape Town, South Africa**

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**Pharmacological investigations of extracts require detailed extract characterization.** This includes botanical characterization of plant material, solvent(s), duration and temperature of extraction, plus other method(s) used for preparation(s). For starting material coming from a company/commercially obtained samples the batch/Lot. Number has to be provided. The drug to extract ratio (DER) must be given. Chromatographic profiling (e.g. HPLC profile with a reference compound recorded at different wavelengths) should be carried out, with at least the major peaks identified, or qualitative and quantitative information on active or typical

constituents should be provided. Altogether the phytochemical standardization of an extract and/or fraction(s) requires stateof-the-art methods.

**4.7.8. Biological screening.** Papers dealing with the biological screening of a meaningful number of extracts of plants or other organisms can be considered for publication in Planta Medica. Identification of the material should properly be documented, and preparation of the extracts should clearly be described (see above, sections **4.7.1** and **4.7.7**). Biological activities should be reported by listing IC50 or EC50 values, or a dose-response relationship should be shown by using at least two test concentrations. Positive controls (reference compounds) should be included. Results should be presented in a concise format, and the discussion should be kept to a minimum.

**4.8. Acknowledgements** should list persons who made minor contributions to the investigation and organisations providing support.

**4.9. Conflict of Interest Disclosure.** A statement describing any financial conflicts of interest or lack thereof is published with each manuscript. The statement should describe all potential sources of bias, including affiliations, funding sources, and financial or management relationships, that may constitute conflicts of interest (please see the ACS Ethical Guidelines to Publication of Chemical Research). The statement will be published in the final article. If no conflict of interest is declared, the following statement will be published in the article: “The authors declare no conflict of interest.”

**4.10. References**, including those in tables and figure legends, should be numbered in the order in which they are cited in the text, using arabic numbers between square brackets, e.g. [1]; for multiple references, e.g. [1–3] or [1, 2,5]. The list of references should be arranged consecutively according to the numbers in the text. Use Index Medicus abbreviations for journal titles. Authors bear complete responsibility for the accuracy of the references. **Original Research articles should not have more than 45 references.**

The following examples illustrate the format for references:

*a) Journals*

Trute A, Nahrstedt A. Separation of rosmarinic acid enantiomers by three different chromatographic methods and the determination of rosmarinic acid in Hedera helix. Phytochem Anal 1996; 7:204–208

Article in press without doi:

Lim EK, Ashford DA, Hou B, Jackson RG, Bowles DJ. Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. Biotech Bioeng, in press

**Note:** If reference is made to papers that are in press, authors are requested to add the galley proof or acceptance letter to the online submission. Avoid references to unpublished personal communications. These have to be included in the body of the text as

“unpublished data”.

Article in press with doi:

Lim EK, Bowles DJ. A class of plant glycosyltransferases involved in cellular homeostasis. EMBO J, advance online publication 8 July 2004; doi:10.1038/sj.emboj.7600295

*b) Books*

Citation to complete book:

Mabberley DJ. The plant book, 2nd edition. Cambridge: Cambridge University Press; 1997: 520–521

Citation to article within a book:

Lechtenberg M, Nahrstedt A. Cyanogenic glycosides. In: Ikan R, editor. Naturally occurring glycosides. Chichester: Wiley & Sons; 1999: 147–191

Lorberg A, Hall MN. TOR: the first ten years. In: Thomas G, Sabatini DM, Hall MN, editors. TOR – target of rapamycin. Heidelberg: Springer Verlag; 2004: 1–18

Multi-volume books and encyclopedias:

Warren SA. Mental retardation and environment. In: International encyclopedia of psychiatry, psychology, psychoanalysis and neurology, Vol. 7. New York: Aesculapius Publishers; 1977: 202–207 Pharmacopoeia of China, Part 1. Beijing: People’s Health Press; 1977: 531–534

*c) PhD and Diploma Theses*

Dettmers JM. Assessing the trophic cascade in reservoirs: the role of an introduced predator [dissertation]. Columbus: Ohio State University;1995

*d) Patents*

Cookson AH. Particle trap for compressed gas insulated transmission system. US Patent 4554399; 1985

*e) Conference Paper*

Okada K, Kamiya Y, Saito T, Nakagawa T, Kaawamukai M. Localization and expression of geranylgeranyldiphosphate synthases in Arabidopsis thaliana. Annual Meeting of the American Society of Plant Physiologists, Baltimore, MD; 1999

*f) Electronic Sources*

Agatep R, Kirkpatrick RD, Parchaliuk DL,Woods RA, Gietz RD. Transformation of S. cerevisiae by the lithium acetate/singlestranded carrier DNA/polyethylene glycol protocol. Technical tips online. Available at http://research.bmn.com/tto. Accessed September 22, 2005

If no author is given, the title is used as the first element of the citation.

**4.11. Chemical structures** should be prepared with ChemDraw or a similar program using the following settings: bond lengths 0.508 cm, bond width 0.021 cm, bold bond width 0.071 cm, bond spacing 18% of length, hash spacing 0.088 cm, atom labels Arial 10, compound numbers Arial 10 bold. These settings correspond to American Chemical Society document settings preset in ChemDraw. The configuration of all stereocenters present should be indicated; use of bold and dashed lines rather than solid and dashed wedges is recommended. They will be reproduced without reduction and the charts should be prepared with maximum widths of up to 8.5 cm for single column print and up to 17.5 cm for double column print. Authors using other drawing packages should modify their program's parameters so that they reflect the above guidelines.

**4.12. Graphics:** Figures are numbered with Arabic numerals. The quality of the illustrations depends on the quality of the originals provided. Graphics cannot be modified or enhanced by the journal production staff. The graphics must be submitted as separate files. The legend should not appear under the figures but should be included as a separate figure legend after the references in the manuscript file. The figure legend needs to be self-explanatory.

Acceptable file formats are TIFF, EPS (vector artwork), or CDX (ChemDraw file). Labeling of all figure parts should be present, and the parts should be assembled into a single graphic.

TIFF files should have the following resolution requirements:

Line Art (Black and White, Color): 800–1200 dpi

| | |
|---|---|
| Greyscale Art: | 600 dpi |
| Color Art (RGB mode): | 300 dpi |

**For efficient use of journal space, single-column illustrations are preferred.**

| | Single (preferred) | Double |
|---|---|---|
| Width | | |
| minimum | 5.5 cm (2.16 in) | 11.5 cm (4.5 in) |
| maximum | 8.5 cm (3.33 in) | 17.5 cm (7 in.) |
| Maximum depth | 25.4 cm (10 in.) | 25.4 cm (10 in.) |

**For best results, illustrations should be submitted in the actual size at which they should appear in the Journal.** Consistently sized letters and labels in graphics throughout the manuscript will help ensure consistent graphic presentation for publication. Lettering should be no smaller than 5 points. (Arial type works well for lettering.) Lines should be exactly 0.5 point. Lettering and lines should be of uniform density. If artwork that should be reduced must be submitted, larger lettering and thicker lines should beused so that, when reduced, the artwork meets the above-mentioned parameters. Complex textures and shading to achieve a three-dimensional effect should be avoided. Different grey scale tones to show group differences are preferred.

**4.13. Tables:** These should be numbered consecutively with Arabic numerals. Tables should be placed in the manuscript or uploaded as separate file (file format DOC, DOCX) after the figure legends. Footnotes in tables should be given lowercase letter designations and be cited in the table by italic superscript letters. The sequence of letters should proceed by line rather than by column. If a footnote is cited both in the text and in a table, insert a lettered footnote in the table to refer to the numbered footnote in the text.

Each table should be provided with a descriptive heading, which, together with the individual column headings, should make the table, as nearly as possible, self-explanatory. Arrangements that leave many columns partially filled or that contain much blank space should be avoided. The table legend should appear directly under the tables.

**4.14. Supporting Information:** To keep articles as concise and at the same time as informative as possible, authors are strongly encouraged to submit part of their tables and figures as Supporting Information. The following type of data will be preferentially published as Supporting

Information rather than in the print article: Spectra, chromatograms, structural drawings outlining NMR correlations, experimental procedures of secondary importance, tables summarizing data that are non-quintessential but useful to the understanding of an article. Tables, figures and text provided as Supporting Information must be referred to in the manuscript as follows: (Table 1S, Supporting Information, etc.). The cover page for Supporting Information should be identical to the cover page of the manuscript. Legends for Figures and Tables must appear directly on the respective figure pages. Pages have to be numbered consecutively. All figures and tables should be referenced in the main manuscript. Supporting Information has to be submitted as a separate file.

Supporting Information is published on the journals homepage at http://www.thieme-connect.de/ejournals/toc/plantamedica.

5. Proofs and Reprints

Galley proofs will be sent to the corresponding author as a PDF file. An electronic author reprint will be supplied free of charge after online publication.

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# APPENDIX C – MTT DATA

## Table C.1: MTT results for ARC 188 after 48 h exposure period

| 48 h<br>Concentration (mg/ml) | % Cell Viability<br>1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 100 | 100 | 100 | 100 | 0 |
| **0.025** | 87.78436004 | 95.05183 | 100.4432 | 94.42647 | 5.186838 |
| **0.05** | 86.22566914 | 87.9502 | 67.16103 | 80.44564 | 9.419979 |
| **0.075** | 92.69158383 | 79.86597 | 108.57 | 93.70919 | 11.74044 |
| **0.1** | 97.50912605 | 104.0697 | 69.82603 | 90.46829 | 14.83998 |
| **0.2** | 86.05767409 | 91.81036 | 84.86844 | 87.57882 | 3.031279 |
| **0.3** | 80.24100342 | 87.96526 | 72.94251 | 80.38293 | 6.133834 |
| **Dead** | 0.732609986 | 0.815702 | 0.795996 | 0.781436 | 0.03545 |

## Table C.2: MTT results for ARC 188 Benz after 48 h exposure period

| 48 h<br>Concentration (mg/ml) | % Cell Viability<br>1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 100 | 100 | 100 | 100 | 0 |
| **0.025** | 90.73856975 | 95.93236 | 88.94908 | 91.87334 | 2.961679 |
| **0.05** | 104.2497069 | 85.25593 | 124.1195 | 104.5417 | 15.86734 |
| **0.075** | 82.24404064 | 92.84161 | 99.92934 | 91.67167 | 7.267235 |
| **0.1** | 85.9971343 | 72.55926 | 68.80211 | 75.78617 | 7.381369 |
| **0.2** | 68.96248535 | 68.8857 | 85.65641 | 74.50153 | 7.887754 |
| **0.3** | 71.74840432 | 50.49977 | 45.72048 | 55.98955 | 11.31272 |
| **Dead** | 0.776236097 | 0.539091 | 0.663651 | 0.659659 | 0.096855 |

**Table C.3: MTT results for ARC 188 fX after 48 h exposure period**

| 48 h | % Cell Viability | | | | |
|---|---|---|---|---|---|
| **Concentration (mg/ml)** | **1** | **2** | **3** | **Average** | **Stdev** |
| **Untreated** | 100 | 100 | 100 | 100 | 0 |
| **0.025** | 118.2920853 | 122.4054 | 124.5368 | 121.7448 | 2.591849 |
| **0.05** | 104.204629 | 115.8358 | 86.11639 | 102.0523 | 12.22799 |
| **0.075** | 93.50315208 | 62.00004 | 80.60781 | 78.70367 | 12.93138 |
| **0.1** | 91.3033793 | 80.43947 | 100.9907 | 90.91117 | 8.394574 |
| **0.2** | 91.53950681 | 76.30965 | 80.47587 | 82.77501 | 6.426595 |
| **0.3** | 106.2195095 | 97.29878 | 65.27834 | 89.59888 | 17.57861 |
| **Dead** | 2.142968524 | 2.37128 | 2.265006 | 2.259751 | 0.093282 |

**Table C.4: MTT results for ARC 2013 after 48 h exposure period**

| 48 h | % Cell Viability | | | | |
|---|---|---|---|---|---|
| **Concentration (mg/ml)** | **1** | **2** | **3** | **Average** | **Stdev** |
| **Untreated** | 100 | 100 | 100 | 100 | 0 |
| **0.025** | 81.63224947 | 82.86375 | 81.79931 | 82.09844 | 0.545441 |
| **0.05** | 102.3200383 | 103.7816 | 85.79046 | 97.29735 | 8.158449 |
| **0.075** | 96.31437612 | 81.60054 | 105.6508 | 94.52189 | 9.899934 |
| **0.1** | 83.31841459 | 86.94949 | 82.2912 | 84.18637 | 1.998322 |
| **0.2** | 94.35551022 | 84.00065 | 95.92523 | 91.42713 | 5.290274 |
| **0.3** | 103.5409468 | 51.61186 | 81.6242 | 78.92567 | 21.28566 |
| **Dead** | 0.937799242 | 0.794308 | 0.718151 | 0.816753 | 0.091065 |

**Table C.5: MTT results for *A. muthi-muthi* gel after 48 h exposure period**

| 48 h | % Cell Viability | | | | |
|---|---|---|---|---|---|
| **Concentration (mg/ml)** | **1** | **2** | **3** | **Average** | **Stdev** |
| **Untreated** | 100 | 100 | 100 | 100 | 0 |
| **0.4** | 97.57067322 | 78.29294 | 81.44024 | 85.76795 | 8.444112 |
| **0.6** | 89.80702777 | 70.42767 | 78.81062 | 79.68178 | 7.935533 |
| **1.3** | 95.57850035 | 78.08173 | 51.02902 | 74.89642 | 18.32619 |
| **Dead** | 0.937799242 | 0.794308 | 0.718151 | 0.816753 | 0.091065 |

**Table C.6: MTT results for *A. muthi-muthi* whole leaf after 48 h exposure period**

| 48 h | % Cell Viability | | | | |
|---|---|---|---|---|---|
| **Concentration (mg/ml)** | **1** | **2** | **3** | **Average** | **Stdev** |
| **Untreated** | 100 | 100 | 100 | 100 | 0 |
| **0.4** | 107.0236063 | 78.86695 | 74.75455 | 86.8817 | 14.34109 |
| **0.6** | 83.69624048 | 69.23823 | 62.60698 | 71.84715 | 8.805075 |
| **1.3** | 79.25392398 | 59.80152 | 60.02656 | 66.36067 | 9.11737 |
| **Dead** | 0.937799242 | 0.794308 | 0.718151 | 0.816753 | 0.091065 |

## APPENDIX D – WOUND CLOSURE DATA

### Table D.1: Wound closure data for ARC 188 after 24 h

| 24 h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 34.79136 | 27.57466 | 29.38856 | 30.58486 | 3.065237 |
| **0.025** | 25.04612 | 30.96509 | 37.18382 | 31.06501 | 4.9557 |
| **0.1** | 21.65 | 39.43312 | 25.49712 | 28.86008 | 7.639457 |
| **0.3** | 18.74624 | 20.47275 | 18.50686 | 19.24195 | 0.875776 |

### Table D.2: Wound closure data for ARC 188 after 48 h

| 48 h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 40.00722 | 37.54655 | 36.39719 | 37.98365 | 1.505851 |
| **0.025** | 26.14194 | 34.99296 | 47.29287 | 36.14259 | 8.673013 |
| **0.1** | 24.12142 | 44.61903 | 25.56247 | 31.43431 | 9.341551 |
| **0.3** | 17.10678 | 21.42807 | 17.61387 | 18.71624 | 1.928697 |

### Table D.3: Wound closure data for ARC 188 Benz after 24 h

| 24 h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 39.12099 | 54.16419 | 56.84125 | 50.04214 | 7.799373 |
| **0.025** | 51.5303 | 63.01503 | 39.38573 | 51.31035 | 9.647874 |
| **0.1** | 38.97101 | 48.1085 | 49.22622 | 45.43524 | 4.593622 |
| **0.3** | 39.08742 | 52.30743 | 47.48263 | 46.29249 | 5.462263 |

**Table D.4: Wound closure data for ARC 188 Benz after 48 h**

| 48 h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 44.7545 | 68.7039 | 66.59824 | 60.01888 | 10.82772 |
| **0.025** | 60.93421 | 74.66175 | 46.15514 | 60.5837 | 11.64041 |
| **0.1** | 43.2918 | 51.38418 | 49.19228 | 47.95609 | 3.417385 |
| **0.3** | 44.81277 | 57.71031 | 51.6665 | 51.39653 | 5.268861 |

**Table D.5: Wound closure data for ARC 188 fX after 24 h**

| 24 h Concentration (mg/ml) | % Wound closure Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 30.88192 | 39.71409 | 35.29801 | 35.29801 | 3.605721 |
| **0.025** | 36.80036 | 34.4176 | 39.18312 | 36.80036 | 1.945516 |
| **0.1** | 16.63271 | 29.89726 | 24.24446 | 23.59148 | 5.434882 |
| **0.3** | 11.63207 | 13.28691 | 18.32 | 14.41299 | 2.844075 |

**Table D.6: Wound closure data for ARC 188 fX after 48 h**

| 48 h Concentration (mg/ml) | % Wound closure Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 37.02412 | 46.40153 | 41.71283 | 41.71283 | 3.828312 |
| **0.025** | 44.59405 | 43.00974 | 46.17837 | 44.59405 | 1.293591 |
| **0.1** | 21.67439 | 35.02672 | 29.84188 | 28.84767 | 5.496211 |
| **0.3** | 11.82819 | 17.01104 | 23.23403 | 17.35775 | 4.662865 |

**Table D.7: Wound closure data for ARC 2013 after 24 h**

| 24h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 56.69183 | 61.37455 | 52.00911 | 56.69183 | 3.823426 |
| **0.025** | 45.29638 | 46.00502 | 28.2154 | 39.83893 | 8.224171 |
| **0.1** | 29.63054 | 44.82479 | 42.91114 | 39.12216 | 6.756904 |
| **0.3** | 20.66283 | 23.04154 | 8.911779 | 17.53872 | 6.17698 |

**Table D.8: Wound closure data for ARC 2013 after 48 h**

| 48h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 64.52193 | 70.06728 | 58.97659 | 64.52193 | 4.527753 |
| **0.025** | 51.31323 | 53.82686 | 32.21222 | 45.7841 | 9.651478 |
| **0.1** | 34.67579 | 49.98813 | 45.61468 | 43.4262 | 6.439931 |
| **0.3** | 23.5908 | 24.69771 | 11.89721 | 20.06191 | 5.790973 |

**Table D.9: Wound closure data for *A. muthi-muthi* gel after 24 h**

| 24 h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 6.782679 | 8.961509 | 7.872094 | 7.872094 | 0.889504 |
| **0.4** | 14.42583 | 15.68814 | 15.9408 | 15.35159 | 0.662691 |
| **0.6** | 15.6369 | 17.83813 | 22.80012 | 18.75838 | 2.995897 |
| **1.3** | 20.12202 | 21.68588 | 18.55816 | 20.12202 | 1.276889 |

**Table D.10: Wound closure data for *A. muthi-muthi* gel after 48 h**

| 48 h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 8.281344 | 10.3599 | 9.32062 | 9.32062 | 0.848565 |
| **0.4** | 15.62271 | 16.68929 | 14.77637 | 15.69612 | 0.782673 |
| **0.6** | 16.45059 | 19.66313 | 25.51677 | 20.5435 | 3.753238 |
| **1.3** | 22.17523 | 21.43294 | 22.91752 | 22.17523 | 0.606077 |

**Table D.11: Wound closure data for *A. muthi-muthi* whole leaf after 24 h**

| 24 h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 65.20539 | 54.18403 | 62.6643 | 60.68457 | 4.71219 |
| **0.4** | 42.04232 | 48.6346 | 55.31187 | 48.66293 | 5.41731 |
| **0.6** | 34.22364 | 46.04925 | 36.83289 | 39.03526 | 5.072744 |
| **1.3** | 33.00092 | 41.77092 | 34.32051 | 36.36411 | 3.860956 |

**Table D.12: Wound closure data for *A. muthi-muthi* whole leaf after 48 h**

| 48 h Concentrations (mg/ml) | Wound closure % Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 97.88384 | 68.45227 | 83.44363 | 83.25992 | 12.01609 |
| **0.4** | 62.772 | 60.86853 | 70.5592 | 64.73324 | 4.192227 |
| **0.6** | 51.17447 | 39.1026 | 46.53034 | 45.60247 | 4.971802 |
| **1.3** | 36.69957 | 48.52817 | 37.7496 | 40.99245 | 5.345778 |

# APPENDIX E – CLOSURE RATE DATA

## Table E.1: Wound closure rate data for ARC 188 after 24 h

| 24 h Concentration (mg/ml) | Closure rate ($\mu m^2/h$) Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 16074.93 | 11907.21 | 18716.3 | 15566.14 | 2802.984 |
| **0.025** | 12852.34 | 16973.81 | 17855.48 | 15893.87 | 2180.603 |
| **0.1** | 11662.31 | 21469.31 | 14812.32 | 15981.31 | 4088.132 |
| **0.3** | 9166.497 | 8047.747 | 8767.444 | 8660.563 | 462.9385 |

## Table E.2: Wound closure rate data for ARC 188 after 48 h

| 48 h Concentration (mg/ml) | Closure rate ($\mu m^2/h$) Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 9242.426 | 8106.618 | 11589.89 | 9646.313 | 1450.435 |
| **0.025** | 6707.326 | 9590.863 | 11354.89 | 9217.694 | 1915.622 |
| **0.1** | 6496.803 | 12146.39 | 7425.143 | 8689.445 | 2473.635 |
| **0.3** | 4182.419 | 4211.64 | 4172.199 | 4188.753 | 16.71316 |

## Table E.3: Wound closure rate data for ARC 188 Benz after 24 h

| 24 h Concentration (mg/ml) | Closure rate ($\mu m^2/h$) Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 25838.47 | 32962.23 | 27605.56 | 28802.09 | 3028.834 |
| **0.025** | 22740.91 | 31967.28 | 19317.99 | 24675.39 | 5342.144 |
| **0.1** | 21455.71 | 22915.74 | 23854.03 | 22741.83 | 986.8044 |
| **0.3** | 18810.64 | 32918.68 | 19345.35 | 23691.55 | 6528.211 |

**Table E.4: Wound closure data for ARC 188 Benz after 48 h**

| 48 h<br>Concentration (mg/ml) | Closure rate ($\mu m^2$/h)<br>Replicates<br>1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| Untreated | 14779.63 | 20905.27 | 16172.07 | 17285.66 | 2621.821 |
| 0.025 | 13445.48 | 18937.8 | 11319.13 | 14567.47 | 3209.9 |
| 0.1 | 11917.27 | 12238.03 | 11918.79 | 12024.7 | 150.8491 |
| 0.3 | 10782.97 | 18159.44 | 10524.97 | 13155.79 | 3539.68 |

**Table E.5: Wound closure rate data for ARC 188 fX after 24 h**

| 24 h<br>Concentration (mg/ml) | Closure rate ($\mu m^2$/h)<br>Replicates<br>1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| Untreated | 13819.19 | 23892.81 | 18856 | 18856 | 4112.538 |
| 0.025 | 15592.08 | 16486.65 | 14697.5 | 15592.08 | 730.4163 |
| 0.1 | 7673.978 | 8677.506 | 12280.87 | 9544.119 | 1978.069 |
| 0.3 | 7112.936 | 6621.361 | 9204.028 | 7646.108 | 1119.746 |

**Table E.6: Wound closure rate data for ARC 188 fX after 48 h**

| 48 h<br>Concentration (mg/ml) | Closure rate ($\mu m^2$/h)<br>Replicates<br>1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| Untreated | 8283.869 | 13958.06 | 11120.96 | 11120.96 | 2316.477 |
| 0.025 | 9480.96 | 10301.22 | 8660.703 | 9480.96 | 669.7376 |
| 0.1 | 5000.053 | 5083.15 | 7558.104 | 5880.436 | 1186.776 |
| 0.3 | 3616.431 | 4238.617 | 5836.426 | 7646.108 | 1119.746 |

**Table E.7: Wound closure rate data for ARC 2013 after 24 h**

| 24 h<br>Concentration (mg/ml) | Closure rate ($\mu m^2$/h)<br>Replicates<br>1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| Untreated | 15344.92 | 16496.314 | 19647.44 | 17162.892 | 1818.641 |
| 0.025 | 18358.43 | 17395.3 | 13857.81 | 16537.18 | 1934.973 |
| 0.1 | 13174.06 | 12377.697 | 17954.85 | 14502.202 | 2462.943 |
| 0.3 | 5137.158 | 9471.1028 | 4608.247 | 6405.5028 | 2178.434 |

**Table E.8: Wound closure rate data for ARC 2013 after 48 h**

| 48 h Concentration (mg/ml) | Closure rate (µm²/h) Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 9024.321 | 9416.3764 | 11139.77 | 9860.1565 | 918.8722 |
| **0.025** | 10398.52 | 10176.436 | 7910.411 | 9495.1213 | 1124.221 |
| **0.1** | 7708.615 | 6901.7389 | 9543.031 | 8051.1282 | 1105.167 |
| **0.3** | 2932.553 | 5075.9306 | 3076 | 3694.8278 | 978.3414 |

**Table E.9: Wound closure rate data for *A. muthi-muthi* gel after 24 h**

| 24 h Concentration (mg/ml) | Closure rate (µm²/h) Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 4515.039 | 6787.556 | 5651.297 | 5651.297 | 927.751 |
| **0.4** | 9039.331 | 10529.43 | 9464.506 | 9677.756 | 626.7421 |
| **0.6** | 11056.66 | 11246.24 | 17094.91 | 13132.6 | 2802.841 |
| **1.3** | 13041.02 | 12663.98 | 13418.06 | 13041.02 | 307.851 |

**Table E.10: Wound closure rate data for *A. muthi-muthi* gel after 48 h**

| 48 h Concentration (mg/ml) | Closure rate (µm²/h) Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 2756.329 | 3923.356 | 3339.842 | 3339.842 | 476.4365 |
| **0.4** | 4894.654 | 5600.69 | 4386.574 | 4960.639 | 497.8523 |
| **0.6** | 5816.007 | 6198.418 | 9565.89 | 7193.438 | 1684.825 |
| **1.3** | 7271.567 | 6258.135 | 8285 | 7271.567 | 827.4643 |

**Table E.11: Wound closure rate data for *A. muthi-muthi* whole leaf after 24 h**

| 24 h Concentration (mg/ml) | Closure rate (µm²/h) Replicates 1 | 2 | 3 | Average | Stdev |
|---|---|---|---|---|---|
| **Untreated** | 41168.96 | 38812.14 | 46824.66 | 42268.59 | 3362.238 |
| **0.4** | 26488.12 | 30872.11 | 35396.03 | 30918.75 | 3636.786 |
| **0.6** | 23089.78 | 36597.26 | 19357.34 | 26348.13 | 7405.686 |
| **1.3** | 19510.33 | 25891.57 | 22632.11 | 22678 | 2605.332 |

**Table E.12: Wound closure rate data for *A. muthi-muthi* whole leaf after 48 h**

| 48 h | Closure rate ($\mu m^2/h$) | | | | |
|---|---|---|---|---|---|
| | Replicates | | | | |
| Concentration (mg/ml) | 1 | 2 | 3 | Average | Stdev |
| **Untreated** | 30900.64 | 24516.26 | 31175.8 | 28864.23 | 3076.532 |
| **0.4** | 19774.27 | 19318.96 | 22576.67 | 20556.63 | 1440.423 |
| **0.6** | 17263.02 | 15538.23 | 12226.9 | 15009.38 | 2089.722 |
| **1.3** | 10848.5 | 15040.02 | 12446.68 | 12778.4 | 1727.182 |

## APPENDIX F – MIGRATION ASSAY DATA

**Table F.1:** **% Migration of HaCaT cells using CytoSelect™ migration assay after 24 h exposure to *A. muthi-muthi* gel relative to an untreated control**

| 24h<br>Concentration (mg/ml) | Migration % Percentage relative to control<br>Replicates | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | Average | Stdev |
| **Untreated** | 100 | 100 | 100 | 100 | 0 |
| **0.4** | 102.6125 | 91.91374 | 76.16876 | 90.23167 | 10.86094 |
| **0.6** | 98.42477 | 103.1322 | 105.1528 | 102.2366 | 2.818773 |
| **1.3** | 91.30093 | 112.7741 | 118.5945 | 107.5565 | 11.73747 |

## APPENDIX G – CALIBRATION PHOTO

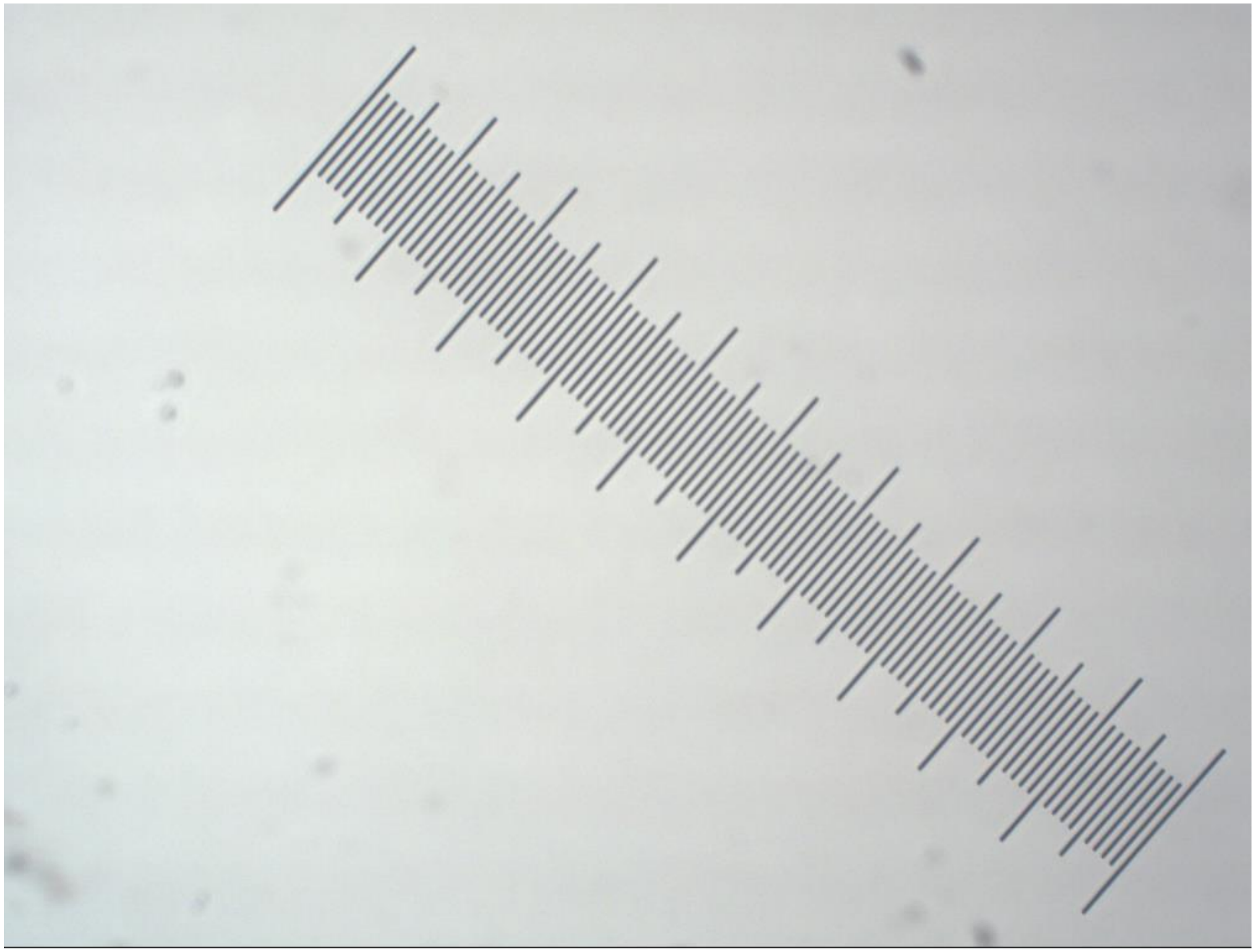

**Figure G.1: Calibration photo used to calibrate a length of 1000 µm at 10 x magnification in ImageJ**