

**ANTIMICROBIAL AND WOUND-HEALING ACTIVITY OF CRUDE
EXTRACTS OF MEDICINAL PLANTS USED IN PARTS OF THE FREE
STATE REGION, SOUTH AFRICA**

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Dissertation submitted in fulfilment of the requirements of the degree

MASTER OF HEALTH SCIENCE: BIOMEDICAL TECHNOLOGY

in the

Department of Health Science
Faculty of Health and Environmental Sciences
at the
Central University of Technology, Free State

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September 2023

DECLARATION OF INDEPENDENT WORK

I, Mamello Sharon Nhlapho, identity number _____ and student number _____, hereby declare that this research project submitted to the Central University of Technology, Free State, for the Master of Health Science in Biomedical Technology degree, is my own independent work; that it complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules, and regulations of the Central University of Technology, Free State; and that it has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

SIGNATURE OF STUDENT

DATE

DEDICATION

My daughter, Kahoentle, who is my rock, inspired me to pursue further education, and I dedicate my work to her. My mother, who finished her master's degree first, is also an inspiration. I hope I have fulfilled my role as your daughter with pride and honour.

ACKNOWLEDGEMENTS

My sincere gratitude to the Almighty Lord for giving me the blessing of starting and completing my work. I thank you, Father.

To my supervisor, Dr Hildah Mfengwana, this study would not have seen the light of day without your support and encouragement and always being the voice of reason when I was losing my way. I cannot thank you enough for your selflessness. May God bless you and enlarge your territory.

Dr Dedré Olivier, who excitedly took me in when I came to enquire about registering for the programme, you have been an incredible help to me in every part of my education, and I am grateful. Please know how much I appreciate it. I pray God's abundant blessing upon your life.

Sincere thanks to Prof. Maryna de Venter and her team for coming through with the laboratory aid for my experiments. I am grateful, Prof.

A special thank you to Dr Frans Maruma, for being an awesome leader and practically forcing me to further my studies. There are few people of your calibre who are gracing the earth.

Big appreciation to my friend, Lesego, for your support in diverse ways and for always calming my nerves.

To my mom, brother, and partner; where would I have been without your constant support, prayers, and love? Thank you. To my baby girl, Kahoentle, you sure did not make this easy for me but we are here now, baby girl.

I would like to thank Ntate Ntheri for availing himself in assisting with the local identification and harvesting of relevant plant parts and Prof. Zietsman for scientific identification of the plants used in this study.

The financial assistance of the National Research Foundation and the Research Committee of the Central University of Technology, Free State, towards this research is hereby acknowledged. I am thankful.

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LIST OF ABBREVIATIONS

(QE)/G	Milligram quercetin equivalent
CFU	Colony-forming unit
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EGFR	Epidermal growth factor receptor
FBS	Foetal bovine serum
FGF-2	Fibroblast growth factor
GAE	Gallic acid equivalent
HaCaT	Human epidermal keratinocytes
HPLC	High-performance liquid chromatography
iNOS	Inducible nitric oxide synthase
JAK-STAT	Janus kinase/signal transducer and activator of transcription
LPS	Lipopolysaccharide
MCF	Michigan Cancer Foundation
MCF-7	Human breast cancer cell line
mg GAE/g	Milligram of gallic acid per gram
MH	Mueller-Hinton
MIC	Minimum inhibition concentration
MMP	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
MZI	Minimum zone of inhibition
NF- κ B	Nuclear factor kappa B
PBS	Phosphate buffered saline
PI	Propidium iodide
RAW 264.7	Mouse macrophage cells
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
TBHP	Tert-Butyl hydroperoxide
TGF- β	Transforming growth factor beta
TLC	Thin-layer chromatography
TNF- α	Tumour necrosis factor alpha

UK	United Kingdom
USA	United States of America
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor

LIST OF UNITS

°C	Degree(s) Celsius
µL	Microlitre(s)
µL/mL	Microlitre(s) per millilitre
cm	Centimetre(s)
g	Gram(s)
m	Metre(s)
mg	Milligram(s)
mg/g	Milligram(s) per gram
mg/mL	Milligram(s) per millilitre
mL	Millilitre(s)
mm	Millimetre(s)
nm	Nanometre(s)
v/v	Volume per volume
µg/mL	Microgram(s) per millilitre

LIST OF SYMBOLS

Ca ²⁺	Calcium ion
CO ₂	Carbon dioxide
dH ₂ O	Distilled water
H ₂ SO ₄	Sulphuric acid
Mg ²⁺	Magnesium ion
NO	Nitric oxide
O ₂	Oxygen

ABSTRACT

INTRODUCTION: A wound develops when the tissue's integrity is damaged, which results in the loss of its protective capabilities. Wound healing is a progressive, overlapping, and complicated process. Microbial infections and excessive free radicals inhibit the advancement of the wound-healing process. Furthermore, available treatments for wounds are accompanied by growing resistance, and because of this there is a continuing search for novel therapeutic options. *Malva parviflora*, *Elephantorrhiza elephantina*, *Pentanisia prunelloides*, and *Dicoma anomala* are some of the medicinal plants that have been used for the treatment of wounds. This research aimed to evaluate the antimicrobial and wound-healing properties of *M. parviflora*, *E. elephantina*, *P. prunelloides*, and *D. anomala*.

METHODOLOGY: The phytochemical screening of *M. parviflora*, *E. elephantina*, *P. prunelloides*, and *D. anomala* was qualitatively and quantitatively conducted using a colorimetric assay. Disc diffusion, nitric oxide (NO) scavenging, and lipoxygenase assays were used to investigate the antimicrobial, antioxidant, and anti-inflammatory properties of crude extracts of *M. parviflora*, *E. elephantina*, *P. prunelloides*, and *D. anomala*. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test was used to assess the cellular proliferation of the four selected plants on human epidermal keratinocytes (HaCaT) cells and their ability to promote cell proliferation, which contributes to wound healing. The tube formation assay was utilised to evaluate the ability of the extract to promote angiogenesis.

RESULTS: The phytochemical screening revealed the presence of flavonoids, saponins, tannins, terpenoids, steroids, and phenols from the methanol and aqueous extracts of *D. anomala*, with the absence of alkaloids, glycosides, and anthraquinones. *E. elephantina* had a strong presence of anthraquinones, saponins, flavonoids, tannins, steroids, terpenoids, and phenols, while alkaloids were not detected. Screened phytochemicals of interest were present from the aqueous and methanol extracts of *P. prunelloides* and *M. parviflora*; however, there was a lack of phenols from *P. prunelloides* and terpenoids from *M. parviflora*. The quantitative estimation of total flavonoids and phenol content showed the *M. parviflora* aqueous extracts to possess most flavonoids, with *E. elephantina* aqueous extracts having more phenols than the other extracts analysed.

All methanol extracts of all the tested plants at low (50 µg/mL) and high concentrations (200 µg/mL) were cytotoxic to the treated HaCaT cells, while the aqueous extracts were not cytotoxic. However, *D. anomala* demonstrated cytotoxicity at low concentrations of 50 µg/mL for the methanol and aqueous extracts; thus excluding it from further investigation for its ability to promote cell proliferation.

The antimicrobial activity assay showed that the aqueous extract of *P. prunelloides* (minimum inhibition concentration [MIC] = 100 µg/mL) and *E. elephantina* (MIC = 50 µg/mL) inhibited the growth of *Candida albicans*, while *M. parviflora* and *D. anomala* showed no antifungal activity against *C. albicans*. The antibacterial results showed the aqueous extracts of *M. parviflora*, *D. anomala*, *P. prunelloides*, and *E. elephantina* to possess antibacterial activity against *Staphylococcus aureus*, *S. epidermidis*, and *Pseudomonas aeruginosa*. The *M. parviflora* extract (MIC = 50 µg/mL and 100 µg/mL) was the most promising extract as it was effective against *P. aeruginosa* (inhibition zone of 28 mm at a concentration of 50 µg/mL), *S. epidermidis* (inhibition zone of 25 mm), and *S. aureus* (inhibition zone of 27 mm) at concentrations of 100 µg/mL.

Antioxidant plant extracts can protect wounds from oxidative damage. The *M. parviflora* leaf extract and whole plant extract had the same antioxidant activities as catechin, which was used as the standard.

E. elephantina showed good anti-inflammatory activity at a concentration of 200 µg/mL by inhibiting the lipopolysaccharide-induced production of NO. However, the *E. elephantina* and *D. anomala* extracts had cytotoxic effects on the HaCaT cells. *P. prunelloides* also indicated promising anti-inflammatory activity by showing low NO production across the concentration of 200 µg/mL, with less cytotoxic effects on HaCaT cells. When analysing the cell viability over the period of 24 hours, both the *M. parviflora* leaf and whole plant extracts displayed no cytotoxic activity on the HaCaT cells, but rather promoted the cell growth.

The results obtained from the tube formation assay demonstrated that the angiogenic factors secreted by the *E. elephantina* extract are capable of inducing tube formation over time after three days. The extract is also able to facilitate the migration and growth of endothelial cells to the wound site to promote wound healing.

CONCLUSION: The findings of this study proved that the investigated medicinal plants – *E. elephantina*, *M. parviflora*, and *P. prunelloides* – can be of medicinal value to humans; however, because of its high cytotoxicity results, *D. anomala* was excluded from the study and not investigated further for wound healing. All extracts, especially the *E. elephantina* extracts, showed promising results due to their antimicrobial and anti-inflammatory activity, as well as their ability to promote the proliferation of cells. The plant also displayed novel findings on the angiogenesis assay by promoting vascular tube formation. This plant can therefore be considered a good candidate for further investigation as a potential source of new active antimicrobial activity compounds for the treatment of microbial infections in wounds and for the promotion of the wound-healing process.

CHAPTER 1:

BACKGROUND

1.1 INTRODUCTION

South Africa boasts a rich diversity of medicinal plants, which for centuries have traditionally been utilised by indigenous communities to treat a range of health conditions and illnesses. Approximately 80% of South Africans continue to rely on medicinal plants for therapeutic purposes due to their affordability, safety, ease of access, cultural acceptability, and patient tolerance (Van Wyk and Prinsloo, 2018). Africa is home to over 25% of the world's plants and approximately 3 000 medicinal plant species that are frequently used in traditional medicine and used for human oral and topical administration (Aremu and Makunga, 2022). Medicinal plant extracts, in particular, are highly sought after as a treatment for skin ailments such as burn wounds, sores, skin ulcers, and skin irritations such as rash, eczema, psoriasis, cancer, tumours, and infectious diseases. This is due to their ability and properties to stop bleeding, promote angiogenesis, and enhance wound healing (Shedoeva et al. 2019).

Physical injuries are the prime factors that cause a break in the epithelial integrity of the skin, which results in wounds and therefore a failure of the function and anatomy of the normal skin tissue. Under normal conditions, skin wound healing starts from the moment an injury occurs. The process of wound healing can be generally categorised into four stages: the haemostasis stage, the inflammatory stage, the proliferate stage, and the remodelling stage. These stages, according to Ghashghaii et al. (2017), involve several steps, which include coagulation, inflammation, formation of granulation tissue, matrix formation, remodelling of connective tissue, collagenisation, and acquisition of wound strength. Capturing the individual processes and functions of wound healing in one experiment is difficult because individual steps in the wound-healing process must be identified and tested separately. Among the many steps of wound healing, the process of cellular migration is considered critical when evaluating wound healing. Understanding this specific cellular function is important for translational medicine that can lead to improved wound-healing outcomes. In the case of this study, the wound-healing properties of specific plants were investigated.

Current antibiotics that are used to prevent the infection of skin wounds include tetracyclines, aminoglycosides, sulfonamides, metronidazole, macrolides, cephalosporins, lyzenoids, and vancomycin (Kaiser et al., 2021); however, high doses of antibiotics can lead to system toxicity (Negut et al., 2018). The treatment of skin wounds and infections using antibiotics can also be expensive and unaffordable to people of low socio-economic status (Yue et al., 2016). It is difficult to discover and develop truly new antibiotics; science is complex, and the research and development process takes a considerable amount of time, which could be costly or in many cases might even fail (Chapman, 2020). In addition, antibiotic-resistant microorganisms have considerably increased due to the overuse and misuse of antibiotics (Anjum et al., 2016).

Medicinal plants have historically been utilised largely by traditional healers to cure health problems, which has prompted scientists to explore the active ingredients in the plants that exhibit therapeutic capabilities and ultimately leading to the discovery of novel medications. Scientists have established the critical methods by which medicinal plants promote healing, inhibit oxidation, and block or influence enzymes, processes, or pathways involved in the development of numerous diseases through various ongoing investigations (WHO, 2023). This is because plant extracts are rich in novel compounds with great medicinal value; thus providing many options for remedies for various chronic medical conditions (Nasim et al. 2022). This study aimed to investigate the antimicrobial and wound-healing properties of the crude extracts of some of the plants used in the Free State region for medicinal purposes.

1.2 JUSTIFICATION OF THIS STUDY

There is currently not enough research on the antimicrobial or wound-healing properties of *Malva parviflora*, *Dicoma anomala*, *Elephantorrhiza elephantina*, and *Pentanisia prunelloides*. The primary emphasis on the bioactivity of these four plants is on conditions such as diarrhoea and gastrobacterial infections rather than wound healing. This study contributes significantly to the discovery of novel natural medicinal antimicrobial agents that can be used successfully for the treatment of wounds.

1.3 AIMS AND OBJECTIVES OF THIS STUDY

1.3.1 Aim

This study aimed to investigate the antimicrobial and wound-healing properties of *M. parviflora*, *D. anomala*, *E. elephantina*, and *P. prunelloides* as medicinal plants used in the Free State region in South Africa.

1.3.2 Objectives

The objectives of the study were as follows:

- To determine the phytochemicals present in *M. parviflora*, *D. anomala*, *E. elephantina*, and *P. prunelloides* plant extracts using the colorimetric assay.
- To evaluate the antimicrobial activities of *M. parviflora*, *D. anomala*, *E. elephantina*, and *P. prunelloides* plant extracts through the disc diffusion assay.
- To investigate the wound-healing potential of *M. parviflora*, *D. anomala*, *E. elephantina*, and *P. prunelloides* plant extracts by using antioxidant, anti-inflammatory, and cellular proliferation assays.

CHAPTER 2:

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter reflects on literature on the anatomy of the skin and wounds and the physiology of wound healing. Factors that affect the physiology of wound healing are also discussed. Thereafter, the focus is on bioactive compounds and active phytochemical constituents that have wound-healing properties. Lastly, the medicinal plants under investigation in this study are discussed.

2.2 ANATOMY OF THE SKIN

The skin is the most important and largest organ in the human body, and it is protected by a composition of layers. It is a complex organ that primarily serves as a barrier against the environment (Pereira et al., 2013), provides resistance to infections, and serves as the first line of defence against pathogens that can invade the body after its integrity has been compromised by cuts, burns, surgical wounds, or trauma (Nester et al., 2004; Tortora & Derrickson, 2010). The three main layers that make up the skin are the epidermis, dermis, and hypodermis. The latter is also known as the subcutaneous layer or the fat layer (see Figure 2.1) (Davis et al., 2005; Török & Conlon, 2005; Weideman, 2007).

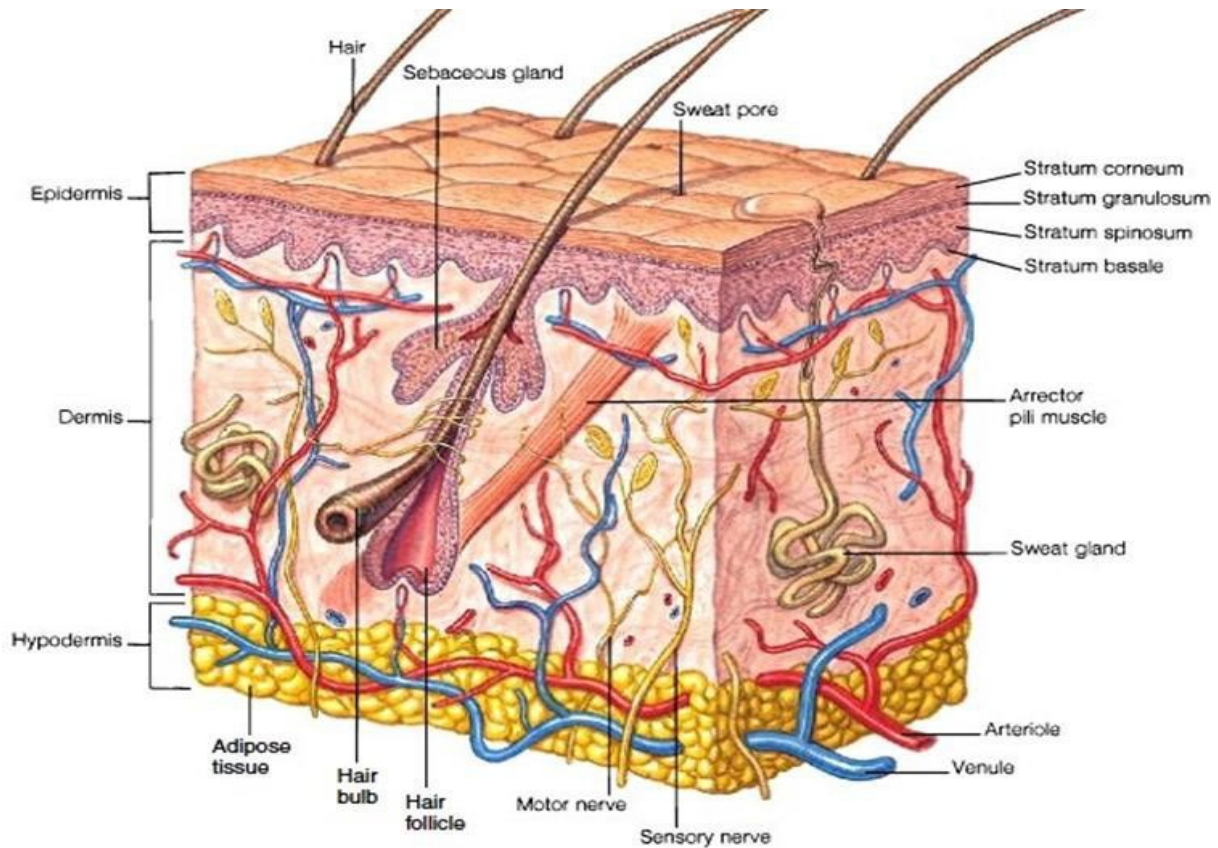


Figure 2.1: Anatomy of the skin, showing the different layers of the skin

Source: Raja et al. (2015)

The epidermis, which is the thin outermost layer, is in direct contact with the environment. It serves primarily to protect the skin from environmental threats such as ultraviolet (UV) light and passive water loss, provides barriers against physical, chemical, and microbial damage, and serves as an innate and adaptive immunologic barrier (Wickett & Visscher, 2006; Dryden, 2010; Fonacier et al., 2010; Baroni et al., 2012). The epidermis comprises five layers, namely the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale (Martini et al., 2013). Within these sub-division layers, the epidermis contains different types of cells, including keratinocytes, melanocytes, Langerhans cells, and occasionally lymphocytes cells. Keratinocytes are the most common type of cell in the epidermis. They are found in the stratum basale (basal layer) and are constantly moving into the stratum granulosum (granular layer), where they change into flat, anucleated, squamous cells called corneocytes (Wickett & Visscher, 2006; Baroni et al., 2012). Keratinocytes generate and express different proteins, such as keratin and filaggrin, which are the main component of the epidermis and make up 80% to 90% of the

structural proteins in the epidermis. Additionally, these cells generate lipids and cytokines, both of which are important in wound healing (Baroni et al., 2012). In the stratum basale, melanocytes produce the skin pigment melanin and defend against UV damage. Langerhans cells in the stratum spinosum play a crucial part in the skin's immunological barrier and contact allergy responses. Finally, the lymphocytes are present under certain conditions, such as during an immunological response (Baroni et al., 2012).

The dermis, which is the layer of skin underneath the epidermis, may be separated into the upper papillary and lower reticular dermis. It is a connective tissue layer that is composed mostly of collagen and elastin generated by fibroblasts that reside inside the layer. The dermis serves to offer mechanical protection, it is responsible for the skin's elasticity, strength, and ability to regenerate with scar formation, and it contains specialised structures such as lymph vessels, sweat and sebaceous glands, and hair follicles (Baroni et al., 2012; McNeil et al., 2014).

The hypodermis comprises the panniculus adiposus (fat layers) and the panniculus carnosus (the vestigial layer of muscle). The hypodermis insulates the body from heat and cold, protects against mechanical stress, and acts as an energy storage area (Török & Conlon, 2005; Weideman, 2007; Dryden, 2010; Martini et al., 2013).

2.3 WOUNDS

Wounds are physical injuries that occur when the skin is broken or an opening occurs, which results in a disruption to its anatomy and function. Wounds can be defined as a breakdown in the protective function of the skin, the loss of continuity of the epithelium, with or without loss of underlying connective tissue following injury to the skin, underlying tissues, or organs (Leaper & Harding, 1998; Strodbeck, 2001; Guest et al., 2017). A wound occurs when the integrity of the skin tissue is compromised through a surgical incision, laceration, abrasion, avulsion, puncture, bite, gunshot, crush injury, contusion or hematoma, friction or shear force, pressure, or as a result of disease, ulcers, or carcinomas (Hirsch et al., 1992; Leaper & Harding, 1998; Mallefet & Dweck, 2008).

Wounds can either be open or closed according to the underlying causes and, depending on how quickly they heal, they are classified as either acute or chronic.

Open wounds are those where the skin has been broken and the underlying tissue is revealed, bleeding is visible, and blood is lost from the body. Examples of open wounds include surgical incisions, laceration wounds, abrasions, puncture wounds, penetration wounds, gunshot wounds, avulsions, bites, cuts, and fish hook wounds. Unlike open wounds, closed wounds do not allow blood to leave the body because the skin is still intact; instead, the blood only leaves the circulatory system. Closed wounds include blood tumours, crush wounds, contusions, and hematomas (Mallefet & Dweck, 2008; Alam et al., 2011; Mittal et al., 2013). Some wounds heal quickly under normal conditions because they follow the usual phases of healing; these types of wounds are called acute wounds.

Acute wounds are defined as tissue injuries that go through the wound-healing process in an orderly manner within the projected period, which results in the proper restoration of the anatomical and functional integrity of the wounded area (Agyare et al., 2016). These types of wounds can be caused by cuts or surgical incisions (Alam et al., 2011). Acute wounds usually show low levels of bacteria, inflammatory cytokines, proteases, and reactive oxygen species (ROS) (Broughton et al., 2006; Velnar et al., 2009).

On the other hand, chronic wounds are associated with reduced oxygenation, chronic inflammation, fibroblast senescence, impaired function levels of critical cytokines, growth factors, and their receptors, abnormal matrix metalloproteinase (MMP) activity, and infection (see Figure 2.2) (Broughton et al., 2006; Velnar et al., 2009). Chronic wounds are injuries that do not heal in the expected time period of four to six weeks or in the usual way as they proceed through the wound-healing process. Chronic wounds are also known as long-term wounds. As a direct consequence of healing slowly, these wounds go into a state of pathological inflammation and either continually reoccur or need a significant amount of time and treatment to heal (Alam et al., 2011; Agyare et al., 2016).

Changes in the profile and activity of cells, abnormal deposition of extracellular matrix components, failure of epithelialisation, and increased levels of free radicals and microorganisms are among the factors that predispose people to develop chronic wounds (Enoch & Leaper, 2008; Guo & DiPietro, 2010). Chronic wounds have high levels of ROS released by neutrophils within the wound environment (Eming et al.,

2007). In addition, infections and conditions such as diabetes mellitus, chronic renal failure, arterial or venous insufficiency, malnutrition, immunodeficiency, and radiation-induced tissue damage are some of the multifactorial processes that further significantly contribute to disrupted wound healing related to chronic wounds (McFarlin et al., 2006; Alam et al., 2011; Brownrigg et al., 2013; Agyare et al., 2016).

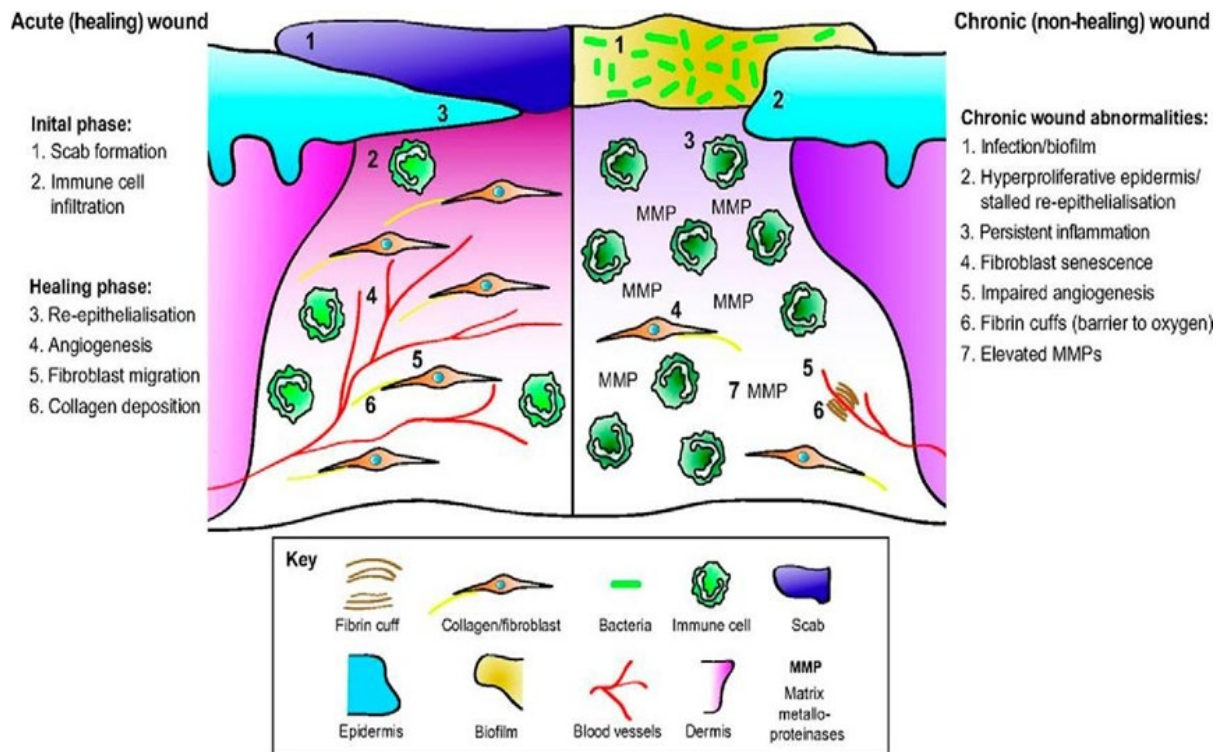


Figure 2.2: Differences between acute and chronic wounds

Source: Nunan et al. (2014)

Wound treatment and maintenance often involve the use of pain medicine, antibiotics, and antibacterial ointment (Reddy et al., 2012). Moreover, wounds that are improperly treated or not treated at all constitute a substantial burden for patients and healthcare systems owing to the expense and length of treatments (Guest et al., 2017). Between 2012 and 2013, there were approximately 2.2 million wound cases in the United Kingdom (UK) alone (Guest et al., 2017). The UK spends approximately £1.94 billion each year on treating an estimated 731 000 leg ulcers and £89.6 million on managing an estimated 87 000 burns and associated comorbidities, respectively. Furthermore, the cost of treating a chronic wound was 135% higher than the cost of treating an acute wound (Guest et al., 2017). In the United States of America (USA), chronic wounds affect 2.8 million people, with an incidence of 120 per 100 000 persons

between the ages of 45 and 65 and increasing to almost 800 per 100 000 in individuals older than 75 years (Nussbaum et al., 2018; Jakucs, 2020). In previous years, the USA spent \$2.3 billion on advanced wound care treatments (Nussbaum et al., 2018). Statistics on the incidence and management of wounds in South Africa are restricted owing to the lack of published wound statistics in the country. Studies by Norman et al. (2007) and Adigun et al. (2010) previously reported that in Nigeria, 32% of all hospital admissions are due to wounds, while in South Africa, 14.3% of all mortality and disability-adjusted life-years are due to wounds. Patients and healthcare systems might benefit from wound-healing-promoting preventive and management identification initiatives.

2.4 INFLAMMATION OVERVIEW

Inflammation is a biological cellular or tissue reaction of the immune system that may be triggered by several causes, including pathogens, infection and injury to cells, toxic chemical irritants or substances, and allergies. It is characterised by heat, oedema, redness, and loss of function. An inflammation response indicates that the body is under threat from a disease or ailment and, as a result, the body prepares to repair the cell or tissue damage. Inflammation occurs when cells such as mast cells, platelets, neutrophils, and monocytes/macrophages produce chemical mediators (prostaglandins, nitric oxide [NO], leukotrienes, vasoactive amines, cytokines-interleukin-1, tumour necrosis factor alpha [TNF- α], and transforming growth factor beta [TGF- β]) in response to an injury, infection, chemical irritation, or allergies (Guo & DiPietro, 2010; Mzindle, 2017; Chen et al., 2018). These mediators are known as pro-inflammatory signalling molecules, and they are the ones that determine how severe the inflammation will be, depending on how long the damage has existed (Chen et al., 2018).

Once they are released into the body, these mediators seek out and bind to specific cell receptors, which, in turn, result in increased vascular permeability, neutrophil chemotaxis activation, an increase in direct enzymatic activity, induced pain, and the promotion of muscular contractions (Iwalewa et al., 2007). Neutrophils clear debris from the wound and guard against bacterial infection by secreting a variety of proteolytic enzymes and ROS (Dunnill et al., 2017). In addition, they produce TNF- α , which boosts neutrophil chemotaxis and makes the subsequent steps of the healing

process easier. The function of macrophages is vital not only during the inflammatory phase of wound healing but also during subsequent stages, such as the proliferation of the various cells that are essential in the healing process. Through their phagocytic activity they also play a role in the debridement and sterilisation of wounds, similarly to neutrophils (Guo & DiPietro, 2010; Dunnill et al., 2017).

In addition, macrophages operate as a source of a variety of growth factors and cytokines that are essential for other processes, such as wound remodelling and angiogenesis (Guo & DiPietro, 2010). Therefore, if inflammation is allowed to continue, these variables have the potential to induce acute and/or chronic inflammatory reactions in the heart, pancreas, liver, kidneys, lungs, brain, gastrointestinal tract, and reproductive system, which might result in tissue damage or illness. Infectious and non-infectious substances, as well as cell injury, may activate inflammatory cells and trigger inflammatory signalling pathways such as the nuclear factor kappa B (NF- κ B), mitogen-activated protein kinase, and Janus kinase/signal transducer and activator of transcription (JAK-STAT) (Chen et al., 2017).

There are three stages of inflammation: the acute transitory stage, the delayed subacute stage, and the chronic proliferating stage. In the first stage, the acute transitory phase, oedema is caused by vascular permeability that results from the inflammatory exudate. This is followed by the migration of leukocytes and phagocytes from the blood into the vascular tissue, which ultimately results in the killing of bacteria, viruses, or parasites and leads to the healing of the wound (i.e., delayed subacute phase). In the third stage, fibrosis develops after the tissue has been degraded (Anilkumar, 2010).

According to Iwalewa et al. (2007), the most significant contributors to inflammation include the migration of leukocytes, production of NO, metabolism of arachidonic acid, ROS, NF- κ B, and pro-inflammatory cytokines. Inflammation may also be triggered by the death of tissues brought on by an inadequate supply of oxygen (O_2) or nutrients. Inflammation may be produced by several different sources in addition to the damage that is done to cells. These conditions include joints, muscles, and damaged bones that have not healed completely. Inflammation is also a potential outcome of a damaged immune system, which may occur when chronic health issues are present.

Another contributor is an imbalance in the ratio of omega-3 to omega-6 fatty acids, which are necessary for protection against illness (Mzindle, 2017).

2.5 PHYSIOLOGY OF WOUND HEALING

Physical injuries are the prime factors that cause a break in the epithelial integrity of the skin, which results in wounds and therefore affect the function and anatomy of the normal skin tissue. Under normal conditions, skin wound healing starts from the moment an injury occurs and lasts for a period of four to six weeks (Shedoeva et al., 2019) depending on the extent of the wound. Wound healing is a complex process that consists of overlapping steps that involve various cell types, signalling molecules, and extracellular matrix components (Pinto et al., 2019). All these components work together to achieve wound healing and restore the body to its normal state. The process of wound healing can generally be categorised into four stages, namely the haemostasis stage, inflammatory stage, proliferate stage, and the remodelling stage. These stages, according to Ghashghaii et al. (2017), involve several steps, which involve coagulation, inflammation, formation of granulation tissue, matrix formation, remodelling of connective tissue, collagenisation, and acquisition of wound strength.

Notwithstanding the source of damage, wound healing follows overlapping processes (see Figure 2.3). Oguntibeju (2019) and Shedoeva (2019) provide a summary of wound-healing phases classically divided into four stages: haemostasis or coagulation, inflammation, proliferation, and remodelling. When a physical injury occurs and Type 1 collagen is exposed, platelets adhering to it are activated, which leads to platelet aggregation by the secretion of glycoproteins. The complex secretes factors that interact with each other to stimulate intrinsic clotting cascade through the production of thrombin, which stimulates the formation of fibrin from fibrinogen. Platelet aggregation, together with fibrin mesh, synthesises a stable haemostatic plug. Following that, the inflammation stage works hand in hand with the haemostatic stage to ensure that blood loss at a wound site is activated. Vasoconstriction of blood vessels and platelet aggregation to induce blood clotting is visible in this stage. Finally, neutrophils migrate into the wound area, engulfing foreign debris and killing bacteria through phagocytosis and releasing proteolytic enzymes (Shedoeva et al., 2019).

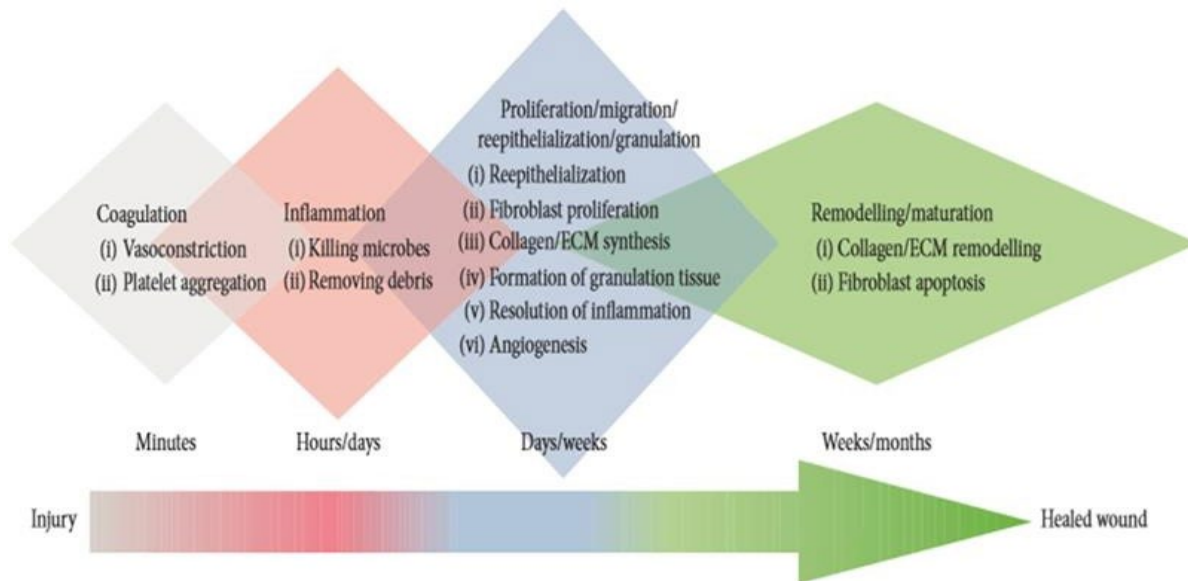


Figure 2.3: Wound-healing cascade

Source: Shedoeva et al. (2019)

The proliferation stage usually starts approximately three days following an injury; this stage is a series of steps that include granulation tissue formation by fibroblast, contraction, and re-epithelialisation by keratinocytes. Granulation involves the role of fibroblast forming collagen with the production of new capillaries that replace the damaged tissue. Thereafter, contraction of the wound occurs when the edges of the wound come together to reduce the wound size. Epithelialisation then forms when keratinocytes migrate, proliferate, differentiate, and reform a functional epidermis, which closes the lesion and protects underlying tissues from further trauma at the wound site. Finally, when the wound matures during the remodelling stage, the collagen cells that had been used to repair the wound, but are no longer needed, are reduced through apoptosis (Shedoeva et al., 2019).

2.6 FACTORS THAT INFLUENCE WOUND HEALING

Even though the wound-healing process progresses naturally, an infection or the presence of excess free radicals can seriously delay this process by prolonging the inflammatory stage, disrupting normal clotting mechanisms, promoting disordered leukocyte function, and delaying angiogenesis (Houghton et al., 2005; Annan & Houghton, 2008).

2.6.1 The role of bacteria and fungi in wound healing

The human skin and mucous membranes often have both pathogenic and non-pathogenic bacteria such as normal microbial flora, which include diphtheroid bacilli (e.g., *Corynebacterium* and *Propionibacterium*) and non-haemolytic aerobic and anaerobic staphylococci (*Staphylococcus epidermidis* and other coagulase-negative staphylococci, rarely *S. aureus*, and *Peptostreptococcus* species). In addition, there are also gram-positive, aerobic, spore-forming bacilli, haemolytic streptococci (*Viridans streptococci*), enterococci, as well as gram-negative coliform bacilli and *Acinetobacter* species that are present on the human skin (Rahim et al., 2017). However, as a result of the unbroken epithelial layer, these bacteria are almost harmless to the human body. Once the skin is injured or a wound occurs, microorganisms that are ordinarily contained at the skin's surface can gain access to the deeper tissues. For a wound to properly heal, the four stages of haemostasis, inflammation, proliferation, and remodelling must occur in the correct order and timeframe. However, bacterial or fungal infection may impede one or more stages of this process, which will result in incorrect or delayed wound healing (Guo & DiPietro, 2010).

It has been observed that bacterial access to the inner tissues of the skin when wounded is one of the most prevalent contributing causes of delayed wound healing (Mustoe, 2004). Typically, bacterial colonisation occurs in wounds due to the absence of an intact epithelial coating, which offers protection against infection and therefore the presence of various bacterial species such as *S. aureus*, *Pseudomonas aeruginosa*, and β -haemolytic streptococci at levels above $10^5/\text{g}$ in wounds plays a central role in delaying healing by producing tissue-destroying enzymes, antiphagocytic and adherence mechanisms, as well as endotoxins and exotoxins (Manjunatha et al., 2015; Martini et al., 2013). As a result, this overwhelms cellular defence mechanisms, which increases the likelihood of wound infection.

One of the purposes of the inflammatory stage of the wound-healing process is to remove any foreign material, including bacteria, from the wound. Consequently, the necessary activation, migration, and proliferation of cells such as neutrophils, fibroblasts, and endothelial cells for timely wound healing must occur (Martini et al., 2013). Bacterial colonisation of the wound may affect the inflammatory response and prolong the healing process. In a wound, the presence of bacteria draws inflammatory cells such as neutrophils. These cells destroy microorganisms using numerous

processes, including phagocytosis, production of ROS, reactive nitrogen species, and proteases such as MMPs (Guo & DiPietro, 2010).

When the bacterial burden grows, so too does the inflammatory activity, which results in a sustained elevation of pro-inflammatory cytokines, proteases, and other components such as ROS (Hoffbrand & Moss, 2011; Guo & DiPietro, 2010; Blakytyn & Jude, 2006). The inflammatory process is therefore prolonged until all contaminated organisms have been eliminated. These substances have the potential to harm healthy body cells and ultimately have unfavourable effects on various parts of the healing process, including the production of granulation tissue, angiogenesis, epithelialisation, collagen, and wound contraction (Levi-Schaffer et al., 1999; Ünlü et al., 2002). The latter allow the wound-healing process to proceed unimpeded.

2.6.2 The role of reactive oxygen species (ROS) in wound healing

ROS are O₂-associated species that have oxidative potential or higher reactivity than molecular O₂ and include singlet O₂, superoxide anion, hydrogen peroxide, and the hydroxyl radical (Soneja et al., 2005). ROS are produced in high amounts by the neutrophils at the wound site during the inflammatory stage, along with other proteolytic enzymes (Houghton et al., 2005). The ROS and proteolytic enzymes serve as antimicrobial defences and aid in the debridement of dead tissue. However, increased numbers of neutrophils cause the release of excess ROS and proteases (Houghton et al., 2005). This overwhelms protease inhibitors that normally protect the tissue cells and the extracellular matrix, resulting in oxidative damage, which kills fibroblasts and other cells and makes skin lipids less flexible (Houghton et al., 2005).

At high concentrations, ROS can induce severe tissue damage and even lead to neoplastic transformation, which decreases the healing process by damage to the cellular membranes, deoxyribonucleic acid (DNA), proteins, and lipids (Süntar et al., 2012). For protection against ROS, human cells generate enzymes, such as superoxide dismutase and catalase, as well as antioxidant compounds such as reduced glutathione (Fukai & Ushio-Fukai, 2011). These antioxidant enzymes and compounds suppress free radicals and ROS, and thus prevent their build-up to toxic proportions. The administration of exogenous substances with antioxidant properties,

such as ascorbic acid, could be a good therapeutic strategy for the prevention of free radical and ROS-induced disease conditions (Gangwar et al., 2014).

2.6.3 The role of antioxidants in wound healing

Antioxidants appear to play a significant role in the successful treatment and management of wounds as they have the ability to reduce the adverse effects of ROS by eliminating products of inflammation (Süntar et al., 2012). Antioxidants counter the excess proteases and ROS formed by neutrophil accumulation at the wound site and protect protease inhibitors from oxidative damage (Houghton et al., 2005). Antioxidant protection by plant extracts or plant compounds is most likely due to the direct interaction between the extracts and the ROS, which limits the damage to cell membranes (Süntar et al., 2012). Plant compounds with high radical scavenging capacity have been shown to facilitate wound healing (Süntar et al., 2012).

2.6.4 The role of prolonged inflammation in wound healing

Even though inflammation is crucial for the process of wound healing, its persistence has been identified as one of the factors that contributes to a delayed recovery period. A normal inflammatory response is an acute reaction that subsides after the triggering stimulus is no longer present (Velnar et al., 2009). However, tissue damage or illness may arise when the normal inflammatory response evolves into a chronic response as a result of long-term inappropriate response to a stimulus or the inability to eliminate offending chemicals (Rondas et al., 2015; Velnar et al., 2009). In addition to having the ability to promote prolonged wound healing, which is often brought on by persistent infection, chronic inflammation also causes a variety of inflammatory diseases such as rheumatoid arthritis, asthma, and inflammatory bowel disease (Baltzis et al., 2014). Fluid derived from chronic wounds that are in the process of healing has decreased levels of cytokines such as TNF- α , interleukin-1 β , and TGF, compared to non-healing chronic wounds (Baltzis et al., 2014). This hints to a link between non-healing wounds and elevated levels of pro-inflammatory cytokines, which would signify a major alteration to the usual inflammatory process in affected patients. According to the findings of previous investigations, chronic wounds are associated with reduced macrophage activity and atypical lymphocyte infiltration (Koh & DiPietro, 2011). Consequently, the release of cytokines and growth factors for the recruitment of

fibroblasts, keratinocytes, and endothelial cells would be hindered in patients with chronic wounds, which would lead to the arrest of healing during the inflammatory stage (Velnar et al., 2009; Koh & DiPietro, 2011; Nunan et al., 2014). Subsequently, anti-inflammatory treatment has been proposed to promote the healing of chronic wounds. In individuals with chronic ulcers who had not responded to other available conventional therapies, infliximab (a TNF- α inhibitor) applied topically led to a substantial improvement in wound healing within four weeks of therapy (Streit et al., 2006). In addition, the wound-healing impact of some plant extracts and plant phytochemicals has been connected to their anti-inflammatory characteristics, and their capacity to promote healing has been ascribed to their ability to lower inflammatory cytokines while boosting anti-inflammatory cytokine levels (Rosique et al., 2015).

2.6.5 The role of angiogenesis in wound healing

Angiogenesis is a multistep process that regulates blood vessel development. It happens in both a healthy and sickly state from birth to death. Angiogenesis is crucial in both physiological (embryonic development, wound healing, and female reproductive systems) and pathological (tumour growth and metastasis) processes (Matutule, 2020). Angiogenesis plays a critical role in wound healing by infiltrating the wound clot and organising into a microvascular network throughout the granulation tissue. This process results in the formation of new blood vessels from existent capillaries (Honnegowda et al., 2015). To maintain angiogenic equilibrium, angiogenesis is regulated by numerous stimulators (pro-angiogenic factors) and inhibitors (anti-angiogenic factors) (Mukherjee & Patra, 2016). Pro-angiogenic factors consist of thrombin, fibrinogen fragments, thymosin- β 4, and growth factors. (Honnegowda et al., 2015). Angiogenesis occurs as an orderly cascade of molecular and cellular events in the wound area where the endothelial cell surface has receptors to which angiogenic growth factors bind in pre-existing vessels (Tsopanoglou & Maragoudakis, 1999; Matsuoka et al., 2006). Following that, growth factor-receptor binding activates signalling pathways within the endothelial cells and the proteolytic enzymes released by activated endothelial cells to dissolve the basement membrane of surrounding pre-existing vessels (Grimm et al., 2009). The endothelial cells then proliferate and sprout outward through the basement membrane and they also migrate

into the wound area site using integrins ($\alpha\beta3$, $\alpha\beta5$, and $\alpha\beta1$), which are cell surface adhesion molecules (Yoshida et al., 2003; Grimm et al., 2009; Nath & Raveendran, 2014). The MMPs dissolve the surrounding tissue matrix in the path of sprouting vessels to allow the vascular sprouts to form tubular channels that connect to form vascular loops (Nguyen et al., 2001). The vascular loops differentiate into afferent (arterial) and efferent (venous) limbs. Finally, new blood vessels mature by recruiting mural cells (smooth muscle cells and pericytes) to stabilise the vascular architecture and blood flow begins in the mature stable vessels.

In contrast to pro-angiogenic factors, anti-angiogenic factors tend to suppress blood vessel growth (Pugh & Ratcliffe, 2003). Some inhibitors circulate in the blood stream at low physiological levels while others are stored in the extracellular matrix surrounding blood vessels. Vascular growth is suppressed when there is a physiological balance between angiogenesis stimulators and inhibitors (Hanahan & Weinberg, 2011). All chronic wounds have decreased angiogenesis, which results in further tissue damage from prolonged hypoxia and poor micronutrient supply; as a result, there is poor formation of new blood vessels and thus decreased entry of inflammatory cells and growth factors such as the fibroblast growth factor (FGF-2) and platelet-derived growth factor, which are essential for wound healing (Pastar et al., 2010; Keswani et al., 2004).

2.7 BIOACTIVE COMPOUNDS / ACTIVE PHYTOCHEMICALS WITH ANTIMICROBIAL, ANTIOXIDANT, ANTI-INFLAMMATORY, AND WOUND-HEALING PROPERTIES

The beneficial properties of medicinal plants lie in the bioactive compounds produced naturally by these plants. These compounds are secondary metabolites such as flavonoids, alkaloids, steroids, tannins, and glycosides, and they are synthesised and stored in selected parts of the plant or in all parts of the plant (Komolafe, 2014). Medicinal plants' bioactive compounds are used as the basis for the production of valuable synthetic compounds such as pharmaceuticals because they are considered as alternative sources of drugs, antibiotics, insecticides, and herbicides (Crozier et al., 2006). This is due to their biological significance and potential health effects such as antimicrobial, anti-inflammatory, and antioxidant activities. The bioactive compounds isolated from medicinal plants have been shown to affect the wound-healing process

(Mittal et al., 2013). They promote and enhance wound repair in a natural way by using a number of mechanisms to induce healing and regenerate lost tissue (Thakur et al., 2011). The activity of bioactive compounds in wound healing can be classified into three categories: phytochemical constituents that contribute to antimicrobial activity, phytochemicals that act as antioxidants and free radical scavengers, and phytochemicals that promote cell proliferation, angiogenesis, collagen production, and DNA synthesis (Hosseinkhani et al., 2017). Antimicrobial characteristics, antioxidant properties, and anti-inflammatory properties contribute to wound healing (Houghton et al., 2005).

2.7.1 Bioactive compounds with antimicrobial and wound-healing properties.

The antimicrobial effects of bioactive compounds can be observed in a study by Ali et al. (2018), which reported that they have antibacterial effects by forming complexes with different extracellular proteins and proteins residing in bacteria cell walls. Bioactive compounds usually act through different mechanisms than conventional antibiotics and could therefore be of use in the treatment of resistant bacteria in wound healing (Borges et al., 2016). Interfering with various growth factors, also known as “stimulators of wound cell”, such as epithelial cells, fibroblasts, and vascular endothelial cells, as well as their regulation, which alters the cellular mechanisms, is how bioactive compounds contribute to the process of wound healing (Schultz et al., 2003). Bioactive compounds are responsible for the regulation of gene expressions and the improvement of skin regeneration owing to the acceleration of cell migration and the deposition of extracellular matrix (Zhang et al., 2012). Alkaloids, flavonoids, tannins, terpenoids, saponins, and phenols are some of the many naturally occurring bioactive compounds that have been investigated for their potential role in wound healing (Bahramsoltani et al., 2014).

Tannins have been demonstrated to possess angiogenesis capabilities, which contribute to the wound-healing process by promoting the migration and proliferation of endothelial cells (Lai et al., 2011). Tannic acid, a polyphenol that has a low level of acidity, is one of the tannins that have received a great deal of attention in the field of wound healing. Tannic acid was used as a cross-linker agent in sponge or hydrogel made of chitosan and/or collagens in a number of studies, and it has been shown that it can generally promote wound closure and increase the rate of wound healing (Moeini

et al., 2020). Additionally, studies have shown that tannins inhibit the growth of many fungi, yeasts, bacteria, and viruses (Samy et al., 2008).

Terpenoids have angiogenic activity, which means that they can simulate growth factors and can be used to treat chronic wounds (Fan et al., 2006). A study conducted by Phaechamud et al. (2015) encapsulated asiaticoside, a triterpene glycoside, in chitosan-aluminium monostearate composite sponge using lyophilisation and dehydrothermal treatment as an absorbent medicinal dressing for chronic wound treatment (Phaechamud et al., 2015). Both normal human dermal fibroblasts and epidermal keratinocytes proliferated in response to the non-toxic sponge (Phaechamud et al., 2015). The investigation on a rat model with a deep partial-thickness burn indicated the beneficial impact of asiaticoside-loaded mats on vascular endothelial growth factors, cluster and proliferating cell nuclear antigens, and the decreased expression of TNF and interleukin-6 (Cao et al., 2016).

2.7.2 Bioactive compounds with antioxidant and wound-healing properties.

Antioxidants seem to play a crucial role in the effective treatment and management of wounds as they have the ability to reduce the negative effects of ROS by removing products of inflammation. This makes antioxidants an important component in the treatment and management of wounds (Süntar et al., 2012). Antioxidants neutralise the excess proteases and ROS that are produced at the wound site as a result of neutrophil accumulation, and they protect protease inhibitors from oxidative damage (Houghton et al., 2005; Süntar et al., 2012). Antioxidant protection by plant extracts or plant compounds is most likely due to the direct interaction between the extracts and the ROS, which limits the damage to cell membranes (Süntar et al., 2012). Plant compounds with high radical scavenging capacity have been shown to facilitate wound healing (Süntar et al., 2012).

Lall and Kishore (2014) classify antioxidants into three classes, namely carotenoids, flavonoids, and polyphenols. An indication of the potential presence of antioxidants in plants includes bioactive components such as tannins, lignans, stilbenes, coumarins, quinones, xanthenes, phenolic acids, flavones, flavonols, catechins, anthocyanins, proanthocyanins, phenols, sterols, flavonoids, nitrogen compounds, vitamins C and E, terpenoids, diterpenoids, phytate, and phytoestrogens (Marwah et al., 2007; Roy et al.,

2011; Albano et al., 2013). These bioactive compounds are responsible for the removal of free radicals, for increasing the contraction of the wound area, and for increasing the formation of blood vessels and fibroblasts (Hosseinkhani et al., 2017). Additionally, these bioactive compounds play a protective role in defending the body from damage caused by a variety of stressors by, among others, absorbing UV radiation and acting as anti-proliferative agents (Kennedy & Wightman, 2011; Saxena et al., 2013). The antioxidative effect of medicinal plants may therefore be mainly due to their bioactive compounds (Süntar et al., 2012).

According to Murugan and Mohan (2012), the primary antioxidant components of natural plant products are phenolic acids and flavonoids. Phenolic compounds are created in plants for a variety of reasons; some of which include the plant's defence against pathogens and other types of environmental stress, including, but not limited to, heat stress, moisture stress, and UV radiation stress (Ncube et al., 2011). They contribute to the maintenance of the human body (Anitha et al., 2012; Sakthidevi & Mohan, 2013; Lall & Kishore, 2014), as well as the prevention of a variety of disorders (Kamatou et al., 2008). Phenolic compounds exhibit anti-apoptosis, anti-ageing, anti-carcinogenic, anti-inflammatory, anti-atherosclerosis, cardiovascular protection, enhancement of endothelial function, suppression of angiogenesis, and cell proliferation activities; among other biological properties (Yadav & Agarwala, 2011) due to the peptides found in plants.

It is believed that flavonoids are non-toxic, and it is noteworthy that they lower the chance of acquiring cancer, cardiovascular problems, obesity, diabetes, ageing-related illnesses, urinary tract infections, and periodontal diseases (Fawole et al., 2010; Gnanaraja et al., 2014). It has also been found that phenolic compounds such as condensed tannins and flavonoids inhibit some molecular targets of pro-inflammatory mediators in inflammatory responses. Because of this, phenolic compounds can be anti-inflammatory or alter the inflammatory processes because they are antioxidants that reduce the number of scavenging radicals (Luseba et al., 2007; Fawole et al., 2010). The redox characteristics of phenolic compounds, which enable them to operate as reducing agents, hydrogen donors, and singlet O₂ quenchers, have metal chelating potential and are largely responsible for their biological activity (Marwah et al., 2007; Kamatou et al., 2008; Shabbir et al., 2013;

Sakthidevi & Mohan, 2013). Consequently, the combination of antimicrobial and antioxidant action promotes wound healing (Pattanayak & Sunita, 2008).

Mittal et al. (2013) reported that sterols and polyphenols have antioxidant properties; as a result, they scavenge free radicals and lower lipid peroxidation, which lowers cell necrosis and increases vascularity. It has also been shown that tannins may scavenge for free radicals and that they possess antimicrobial characteristics, both of which promote the healing process of wounds (Ghosh & Gaba, 2013).

2.7.3 Bioactive compounds with anti-inflammatory and wound-healing properties.

Flavonoids, triterpenoids, sterols, tannins, alkaloids, chalcones, anthraquinones, sesquiterpenoids, curcumin, coumarins, polyphenolic compounds, carotenes, and limonoids (Okem, 2011) are plant bioactive compounds that have been found to have the ability to act as anti-inflammatory and wound-healing agents and are capable of modifying the activities of inflammatory cells (Iwalewa et al., 2007). They have also been used in the cosmetics sector for their anti-inflammatory properties (Lall & Kishore, 2014). Bioactive compounds, particularly flavonoids and terpenoids, are known to promote the wound-healing process by contracting the wound site and increasing the rate of epithelialisation, tensile strength, and hydroxyproline content (Ghosh & Gaba, 2013; Mittal et al., 2013; Pawar & Toppo, 2015). Additionally, flavonoids have been shown to inhibit the secretion of pro-inflammatory cytokines such as interleukin-6, increase the secretion of interleukin-10 under various conditions (Thring et al., 2011), and some act as phospholipase inhibitors that inhibit the cyclooxygenase and lipoxygenase enzymes involved during the inflammatory phase (Fawole et al., 2009; Agnihotri et al., 2010). Certain flavonoids may be utilised to treat inflammatory pain conditions since they have been demonstrated to have comparable actions as synthetic non-steroidal anti-inflammatory drugs (Iwalewa et al., 2007). They are also thought to inhibit the production of histamine by basophils and mast cells, which are inflammatory cells (Mzindle, 2017).

Tannins play a major role in wound healing by acting as astringents mainly for wound contraction and increasing the rate of epithelialisation (Tsala et al., 2013). Tannins also induce a local anti-inflammatory impact by promoting capillary

vasoconstriction, which in turn reduces vascular permeability. This is another way that tannins exert their anti-inflammatory influence (Edwin et al., 2009).

According to Ameyaw and Duker-Eshun (2009), alkaloids contain substantial biological activity, including antimalarial, antibacterial, antihyperglycemic, anti-inflammatory, and pharmacological properties. It is believed that they accelerate the process of wound healing in its early stages by accelerating the migration of fibroblasts to the location of the wound (Tsala et al., 2013). Mittal et al. (2013) reported that saponins are responsible for wound contraction, as well as a high rate of epithelialisation, due to its antioxidant and antimicrobial activity.

2.7.4 Bioactive compounds with angiogenesis and wound-healing properties

The phytochemical constituents such as flavonoids, sterols (β -sitosterol), anthraquinones, tannins, and saponins (ginsenosides Rg1, Re, Rg2) play a significant role in the process of angiogenesis through various signalling pathways. The flavonoids in research by Majewska and Gendaszewska-Darmach (2011) are said to stimulate the migration of human umbilical vein endothelial cells, they have demonstrated strong angiogenic activity on the chorioallantoic membrane of chick embryos and stimulate neo-vascularisation in the mouse matrigel plug assay. The flavonoids are also said to enhance new vessel formation in a gerbil brain damaged by ischaemia/reperfusion, especially in the cingulate cortex and septal regions, in a dose-dependent fashion. Lastly, flavonoids enhance the expression of proteins related to angiogenesis, Von Willebrand factor (vWF), vascular endothelial growth factor (VEGF), VEGF receptor Flk-1, and blood vessel matrix laminin (Chang et al., 2002).

A study by Li et al. (2011) that investigated the wound-healing properties of tannin extracts from immature fruits of *Terminalia chebula* Fructus Retz. revealed that the tannin extracts could up-regulate immune-histochemical, transcriptional, and translational levels of VEGF-A expression; thus increasing the amount of newly formed capillaries at the inflammatory phase, as well as the percentage of wound contraction at the granulation formation and scar remoulding phases. They also found that the tannin extract had the strongest angiogenic effect when compared to ointment or Vaseline applied to the wound area in the tested rats.

Saponins from panaxatriol saponins in a study by Hui et al. (2017) were able to increase capillary densities and enhance angiogenesis in ischaemic boundary zones and up-regulate VEGF and Angiopoietin-1 expression.

2.8 THE USE OF MEDICINAL PLANTS FOR WOUND CARE

Subrat (2002) estimated the global market for traditional medicines to increase at an annual rate of 20%. Since natural resources are being harvested from the wild in a manner that is not ecologically sound, the number of traditional medicinal plant species that are in danger on a global scale ranges between 4 160 and 10 000 (Schippmann et al., 2002). Approximately 80% of black South Africans utilise traditional medicine every day as their source of healthcare treatment, which amount to around 26.6 million users (Xaba, 2016). Traditional medicine is essential for the treatment of a wide variety of ailments, including those that Western medicine may not be able to adequately cure. Native plants serve as the primary source of traditional medicine (Kong et al., 2003; Rao & Arora, 2004; Shai et al., 2008).

Approximately 3 000 indigenous plant species are used as medicine by South African traditional healers (De Wet et al., 2013). Medicinal plants play an important role in the treatment of skin ailments (Weideman, 2007; De Wet et al., 2013), and several plants have been investigated for the treatment and management of skin ailments (Hutchings et al., 1996; Grierson & Afolayan, 1999; Mabona & Van Vuuren, 2013; De Wet et al., 2013). The phytochemical constituents that medicinal plants naturally generate are what give them their therapeutic benefits. These phytochemicals, which include secondary metabolites such as flavonoids, alkaloids, steroids, tannins, steroids, anthraquinones, and glycosides, are synthesised and preserved in either certain plant sections or across the entire plant (Komolafe, 2014).

Many medicinal plant extracts inhibit NO and ROS generation. Firdous and Sautya (2018) reported that medicinal plants demonstrate several wound-healing mechanism potentials by favouring pro-inflammatory cytokines by the activation of interleukin-8, increased expression of inducible nitric oxide synthase (iNOS) and alpha-1 type I collagen, and antimicrobial activity. Medicinal plant extracts were reported to contribute to wound healing by increasing VEGF, which is responsible for angiogenesis, which is a process that is necessary in the remodelling stage of wound

healing. Additionally, the expression of TGF- β is increased, which in turn acts in the regulation of cell proliferation. TGF- β causes the migration of leukocytes into injured tissue, which results in the formation of granulation tissue (Firdous & Sautya, 2018).

Furthermore, the aqueous extract of Korean red ginseng stimulates in vivo and in vitro angiogenesis through the activation of the PI3K/Akt pathway required for the migration of skin epithelial cells in response to wounds (Firdous & Sautya, 2018). Extracts of *Caesalpinia ferrea*, according to Pereira et al. (2016), greatly increased the expression of iNOS, which regulates the formation of collagen, cellular proliferation, and wound contraction. The expression of alpha-1 type I collagen from the topical application of the ointment of *Delonix elata* contributes to wound healing (Firdous & Sautya, 2018). Since microbial access to and infection of the interior tissues of the injured skin impede wound healing (Mustoe, 2004), it is important to investigate the antimicrobial activity of plant extracts.

2.9 MEDICINAL PLANTS INVESTIGATED IN THIS STUDY

2.9.1 *Pentanisia prunelloides*

The gardenia family (Rubiaceae), which encompasses 650 genera and 13 000 species, includes *P. prunelloides*. *Pentanisia* is taken from the Greek word *penta*, which means five, and *anisos*, which means uneven, alluding to the calyx lobes, and *prunelloides*, which is similar to the genus *Prunella* (Lamiaceae), which is derived from the Latin *prunus*, which means purple. There are 15 species of *Pentanisia*, three of which are found in South Africa. *P. prunelloides* is a tall, upright plant with thick, hairy stems sprouting from its tuberous roots, reaching a height of around 600 mm, and is found throughout the eastern grasslands of South Africa (Siebert et al., 2002; Yff et al., 2002). The plant can resist fires and dry winter months because of its large root system. The delicate purple flowers of this plant bloom throughout the early summer months of August to January (see Figure 2.4). On the blooms, four or five petals create a tube. The fruits of this family are often capsules, berries, or drupes (Germishuizen & Clarke, 2003).



Figure 2.4: *P. prunelloides* flowers and whole plant

Source: South African National Biodiversity Institute: PlantZA (2004)

P. prunelloides is an important medicinal plant in South Africa because of its multipurpose properties, which include the treatment of several internal and external ailments such as boils, burns, snake bites, swelling, rheumatism, fever, chest pains, toothache, ulcers, and venereal diseases (Mpofu et al., 2014). The fresh roots of this plant may be chewed and swallowed for relief of heartburn; incidentally, the plant's Sesotho vernacular name is setima-mollo, which means “fire extinguisher” in English (Moteetee & Van Wyk, 2011). Root decoctions may be administered orally for diarrhoea and indigestion, while leaf decoctions taken orally three times a day may treat diarrhoea as well (Moteetee & Van Wyk, 2011). Vomiting is treated with the root that is dried and grated, which is taken orally.

P. prunelloides is used extensively in combinations with *E. elephantina* to treat various ailments. An ethnobotanical survey of traditional medicinal plants used against lymphatic filariasis by Komoreng et al. (2017) revealed that the leaves and underground parts of *P. prunelloides* are used often and are thus in high demand. Methanol and aqueous extracts of the rhizomes of *P. prunelloides* and two of its active bio-compounds showed antimicrobial activity against gastrointestinal bacteria (Mpofu et al., 2014).

2.9.2 *Elephantorrhiza elephantina*

The genus *Elephantorrhiza* (see Figure 2.5) is an exclusively African genus and the name was first published in 1841 by Betham (Smith, 1966). It belongs to the Fabaceae

family, which contains 13 species (Mpofu, 2012). The name is derived from the Greek word *elephantorrhiza*, which means “elephant root”. *E. elephantina* is also known as *intolwane* (in isiXhosa and isiZulu) and *mositsane* (in Sesotho and Tswana) (Phillips, 1917; Jacot Guillarmod, 1971). *E. elephantina* is the most widely distributed and most commonly encountered of the species found in this family. This plant flourishes in hot, dry grassland areas with open space and is endemic to Africa south of the equator, including Angola, Namibia, Botswana, Zimbabwe, Mozambique, and almost all areas of KwaZulu-Natal, Limpopo, North West, Mpumalanga, Free State, Northern Cape, and Eastern Cape, as well as Eswatini and Lesotho (Ross, 1974).



Figure 2.5: *E. elephantina* leaves

Source: Researcher (2020)

E. elephantina has a large woody rootstock from which grow numerous unbranched leafy shoots of limited growth of up to 10 cm high (Van Wyk, 2002).

E. elephantina twigs grow directly from the soil, and it has an enormous root of about 8 m long. This plant has dull green leaves, which are bipinnately compound with two to 17 opposite or sub-opposite pairs of pinnae. The flowers are arranged in axillary, solitary, or clustered racemes that are golden yellow to pale yellowish white in colour and near the ground level. The plant blooms between September and December.

The Fabaceae family is the most commonly used family for medicinal purposes (Van Wyk, 2002). The rhizome of *E. elephantina* is used extensively in traditional medicine

to treat a wide range of ailments, including fever, ulcers, diarrhoea, dysentery, dysmenorrhoea (Bryant, 1966), chest problems, heart conditions (Watt & Breyer-Brandwijk, 1962), syphilis, and bleeding (Jacot Guillarmod, 1971). It is also used to remedy haemorrhoids and perforated peptic ulcers, as well as for an emetic and dermatological purposes (Hutchings et al., 1996; McGraw & Eloff, 2008). The grated root is usually steeped in water for 24 hours or more, after which it is strained before being administered externally. The infusion is boiled for approximately 10 minutes and small quantities are taken three times a day for internal uses. The face is held in the vapour of a warm infusion to treat acne (Van Wyk, 2002).

2.9.3 *Malva parviflora*

M. parviflora (see Figure 2.6) is a widespread tropical plant that belongs to the Malvaceae family (Ridh et al., 2018). It is commonly known as cheese weed but has a South African vernacular name, tika-motse, from the Basotho people. The plant has two distinct growth forms: it may be prostrate, branching flat on the ground or in an upright growth. It is an annual or perennial herb that grows up to 50 cm in height, and has a deep strong tap root system that makes it suitable to grow in sandy (light), medium (loamy), heavy (clay), and well-drained soils (Raina & Sangeeta, 2017). The leaves are dark green, broad, and have five to seven rounded lobes that are 8 to 10 cm in diameter. *M. parviflora* has distinguishable small white or pink flowers with 4- to 6-mm-long petals (Agyare et al., 2013; Raina & Sangeeta, 2017).



Figure 2.6: *M. parviflora* leaves

Source: Researcher (2020)

M. parviflora is a remarkable gift from nature to mankind. The plant has proven to have significant pharmacological potential and can be used in the treatment of different ailments. The flowers and leaves are soothing and used for softening sensitive areas of the skin and in the management of wounds and swelling. An ointment made from the leaves of *M. parviflora* can be used to treat bruises and broken bones and to drain swollen, inflamed purulent wounds. Additionally, the leaves help to reduce gut irritation and have a laxative effect. Root or leaf decoctions of the plant may be used to remove dandruff and to soften hair. *M. parviflora* has also previously been used to treat headaches, fever, sores, and digestive problems and may be applied as a dressing to reduce swelling and draw out toxins. *M. parviflora* extracts contain different amounts of phenols, flavonoids, saponin, alkaloids, resin, and tannins (Navneet, 2017).

Pharmacognostic studies of *M. parviflora* have revealed that the whole plant and different parts are used in the treatment of various illnesses. The flowers and leaves of this plant are soothing and used for softening of sensitive areas of the skin (Mabona et al., 2013). *M. parviflora* has been reported to have antibacterial (Shale et al., 2005), antidiabetic (Gutierrez, 2012), and antifungal (Wang et al., 2001) properties.

M. parviflora leaf extracts have also been proven to be anti-inflammatory (Lagunas-Herrera et al., 2019; Ramírez-Serrano et al., 2019), analgesic (Afolayan & Sunmonu, 2010), antioxidative (Bouriche et al., 2011), and neuroprotective (Aslam & Sial, 2014). The leaves are used in draining swollen, inflamed purulent wounds (Afolayan & Sunmonu, 2010). It is evident that researchers have proved the pharmacological potential of *M. parviflora* in treating different ailments of a suppurative and non-suppurative nature.

2.9.4 *Dicoma anomala* Sond.

D. anomala (see Figure 2.7), which belongs to the Asteraceae family, was originally described by Cassini in 1817 (Maroyi, 2018). It comprises 50 species, of which Southern Africa is home to 16 of those species (Maroyi, 2018). The genus name *Dicoma* is derived from the Greek words *di*, which means two, and *kome*, which means tuft of hair, in reference to the species' double row of pappus bristles (Mnegwane & Koekemoer, 2007). The species name *anomala* is derived from Latin and means uneven or abnormal (Mnegwane & Koekemoer, 2007). Ortiz and Rodriguez-Oubina (1996) determined that *D. anomala* is a single complex species comprising three distinct subspecies: *D. anomala* Sond. subsp. *anomala*, which is widespread; *D. anomala* Sond. subsp. *attenuata* (Ortiz & Rodriguez-Oubina, 1996), which is endemic to eastern central Angola, the Democratic Republic of the Congo, and Zambia (Foden & Potter, 2005). *D. anomala* is also found in Botswana, Burundi, Lesotho, Malawi, Mozambique, Rwanda, Tanzania, Uganda, and Zimbabwe (Maroyi, 2018).



Figure 2.7: *D. anomala* Sond. roots (A) and leaves (B)

Source: Researcher (2020)

D. anomala is found at elevations ranging from 165 to 2 075 m in rocky grasslands, slopes, or flat grassland, as well as in savannah on doleritic or sandy soils (Mnegwane & Koekemoer, 2007). *D. anomala* is an upright, suberect, or prostrate perennial plant with fragrant semi-woody tubers at the base of a subterranean woody stem. It may have a few to several hairy, upright stems that emerge from a woody rhizome. The stems vary in length from 5 cm to 60 cm, are branching, yellowish, and trail over the ground and over nearby vegetation and rocks (Pope, 1992). The leaves are simple, thin, alternately arranged on the stem, stalkless, and linear or narrowly lanceolate in form. The top surface of the leaves is glabrous, olive green, or sometimes grey, rough, and characterised by a prominent central vein along which the leaf folds inward. The underside is white and hairy, with a little irregular border. The terminal flower heads are cup or cone shaped and cream to pinkish white in colour (Maroyi, 2018).

The medicinal uses of *D. anomala* can be linked to several pharmacological properties such as anthelmintic (Mølgaard et al., 2001), antihyperglycemic (Balogun & Ashafa, 2017; 2018), anti-inflammatory (Shale et al., 1999), antibacterial (Mabona & Van

Vuuren, 2013; Steenkamp et al., 2004), antioxidant (Munodawafa et al., 2013; Mbhele et al., 2015; Balogun et al., 2016), antiparasmodial (Tselanyane, 2006), hepatoprotective (Balogun et al., 2016), anticancer, and wound healing (Van der Merwe, 2008). Ethnomedicinal information has been found in Botswana, Lesotho, Malawi, Namibia, South Africa, Eswatini, Tanzania, and Zimbabwe, which represent 57.1% of the countries where *D. anomala* is indigenous (Maroyi, 2018). Ranked first is Lesotho, with 32 ethnomedicinal uses and 13 literature recordings, followed by Zimbabwe with 23 uses and eight literature records, Malawi with 13 uses and three literature records, and Namibia with nine uses and a single record.

The majority of human and animal ailments and diseases, including diarrhoea, dysentery, stomach problems, pain, sores and wounds, colds, cough, sore throat, sexually transmitted infections, blood circulation problems, fever, malaria, and are treated with concoctions prepared from *D. anomala* and traditional ethnoveterinary medicine. The roots of the plant may be used for coughs and chest ailments, to strengthen blood after difficult labour in mothers, as well as for back pain and stomach problems. The leaves and twigs can be used for the treatment of cancer and high blood pressure. The roots also may be used for wounds, sores, and skin ulcers (Van der Merwe, 2008). A study by Steenkamp et al. (2004) reported on the evaluation of *D. anomala* roots for their antibacterial and anti-inflammatory properties, where methanol and aqueous extracts of the plant were found to exhibit activity against all bacteria tested.

D. anomala is also used in combination with other plant species; for instance, in Lesotho, *D. anomala* root infusions are taken orally combined with *Berkheya setifera* DC roots as a cure for bilious attacks (Watt & Breyer-Brandwijk, 1962). For painful menstruation, the root infusion of this plant is administered orally in combination with *Scabiosa columbaria* L. (Jacot Guillarmod, 1971; Moteetee & Van Wyk, 2011). For venereal disorders, the root infusion of this plant is used orally in combination with *Helichrysum caespititium* (DC.) Sond. ex Harv., *S. columbaria*, and *Zantedeschia albomaculata* (Hook.) Baill. As a treatment for gall disease, the root infusion of this plant is administered in combination with *Cymbopogon* spp. (Jacot Guillarmod, 1971; Moteetee & Van Wyk, 2011). In Malawi, *D. anomala* root infusion is used orally with *Trichodesma physaloides* (Fenzl) A.DC. roots to treat backache (Gelfand, 1985). In Malawi and Zimbabwe, a root infusion of *D. anomala* is given orally in combination

with soot and the roots of *Aspilia pluriseta* Schweinf to induce labour, guarantee an uncomplicated delivery, and aid in the ejection of the placenta and cleansing of the uterus after childbirth (Gelfand, 1985).

The medicinal plants in this study were subjected to the methodologies discussed in the next chapter to achieve the aims and the objective of the study. Thereafter, the results obtained are discussed and analysed to provide concluding results on the wound-healing and antimicrobial properties of these plants.

CHAPTER 3: METHODOLOGY

3.1 INTRODUCTION

This chapter outlines the methods used to achieve the aim and objectives of this research study. The plant selection, collection, and identification, and the preparation of the plant material to crude extracts are discussed.

3.2 PLANT SELECTION

Different academic search engines and publications were used to identify articles on the medicinal plants commonly used for the treatment of wounds and the most common plants that were found to have wound-healing properties as per ethnobotanical claims were *M. parviflora*, *D. anomala*, *E. elephantina*, and *P. prunelloides*.

3.3 PLANT MATERIAL COLLECTION AND IDENTIFICATION

Plant species were collected from Thaba 'Nchu in the Free State province of South Africa, with the assistance of a herbalist. The herbalist provided the local identification of the plants, while the scientific identification of the plant was done by Prof. Zietsman from the Botany Department of the National Museum, Bloemfontein. Voucher specimens EE2022CUT, PP2022CUT, DA2022CUT, and MP2022CUT were prepared and deposited at the Herbarium of the Free State National Botanical Garden in Bloemfontein.



Figure 3.1: Harvesting of plant material in Thaba 'Nchu, Free State

Source: Researcher (2020)

3.4 PREPARATION OF PLANT MATERIAL

Once collected, the plants were washed with distilled water (dH_2O) to get rid of any foreign materials and soil present and they were air-dried for 30 days at room temperature ($\sim 20^\circ\text{C}$). Once the plants were dry, they were ground into a fine powder using a grinder and kept in marked Schott bottles in a cool, dark area until they were used. Before usage, the twigs, leaves, and flowers of *M. parviflora* were mixed to

prepare an extract that is made up of the whole plant and another extract was prepared using only the leaves of the plant. For the other plants, only the roots were used to prepare the extracts. The selection of plant parts used in the study was prompted by prior research that has been conducted on the ethnobotanical uses of the selected plants due to their antimicrobial and wound-healing properties.

Additionally, the herbalist who assisted with the identification of the plants during harvesting gave information on the part of the plant that is to be utilised.

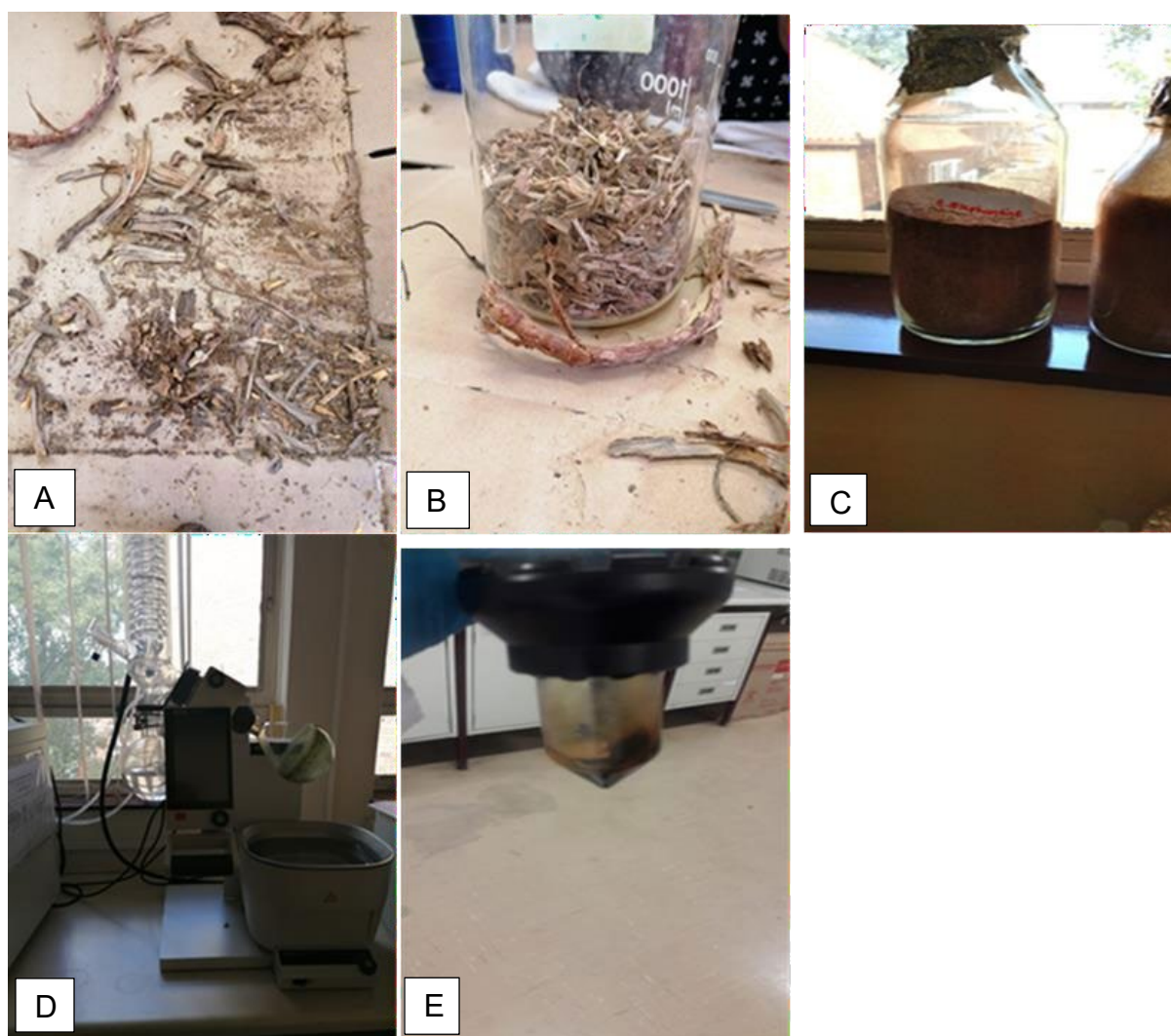


Figure 3.2: Steps in the preparation of the plant extracts: A) Drying the plant material; B & C): Grinding and powdering the plant material; D) Concentration of methanol extracts; E) Freeze drying the aqueous extracts.

3.5 PREPARATION OF EXTRACTS

The maceration method, as adopted from Jeremy and Whiteman (2003), with minor modifications, was used to extract the plant material. An amount of 50 g each of the powdered plant material was extracted with 500 mL methanol and dH₂O respectively and left on a shaker for 72 hours (Jeremy & Whiteman, 2003). Thereafter, the extracts were filtered using Whatman No. 1 filter paper. The methanol filtrates were concentrated using a rotary evaporator and the aqueous extracts were lyophilised using a freeze dryer.

3.6 PHYTOCHEMICAL ANALYSIS

With slight modifications to the methodologies and procedures of Harborne (1973), Edeoga et al. (2005), Sofowora et al. (2013), Chandrashekar et al. (2011), and Al Ghasham et al. (2017), the methanol and aqueous extracts of *M. parviflora*, *E. elephantina*, *P. prunelloides*, and *D. anomala* were subjected to qualitative phytochemical screening. The presence of alkaloids, flavonoids, terpenoids, saponins, anthraquinones, steroids, glycosides, and tannins was determined individually.

3.6.1 Qualitative phytochemical analysis

3.6.1.1 Test for alkaloids

An amount of 0.2 g of the crude plant extract was dissolved in 2 mL of 1% aqueous hydrochloric acid, heated on a water bath, and filtered. One millilitre (1 mL) of the filtrate was then treated with 1 mL of Mayer's reagent and a second portion was treated with 1 mL of Dragendroff's reagent. The generation of orange precipitate was taken as preliminary evidence of the presence of alkaloids in the extract (Harborne, 1973).

3.6.1.2 Test for flavonoids

One gram (1 g) of the crude plant material was heated with 2.5 mL ethyl acetate over a steam bath for three minutes and allowed to cool down. The mixture was then filtered using 47-mm Whatman No. 1 filter paper and was shaken vigorously on a vortex for one minute with dilute 0.25 mL of ammonia solution. The formation of a yellow precipitate indicated the presence of flavonoids (Sofowora, 1993).

3.6.1.3 Test for terpenoids

An amount of 0.2 g of plant extract was diluted in 1 mL of chloroform and 3 mL of sulphuric acid (H_2SO_4) was carefully added to produce a layer. The interface was reddish brown in colour, which indicated the presence of terpenoids (Harborne, 1973).

3.6.1.4 Test for saponins

An amount of 0.5 g of crude extract was boiled for two minutes in 5 mL of dH_2O in a water bath and then filtered. Thereafter, 5 mL of the filtrate was mixed with 3 mL of dH_2O and then shaken vigorously by hand for two minutes and observed for a stable, persistent froth. The frothing was then mixed with three drops of olive oil, shaken, and observed for the formation of an emulsion as an indication of saponins (Harborne, 1973).

3.6.1.5 Test for anthraquinones

An amount of 0.2 g of the extract was boiled for five minutes in 4 mL of 98% H_2SO_4 and then filtered while still hot. Thereafter, 2 mL of chloroform was added to the filtrate and shaken, whereafter 1 mL of ammonia was pipetted into another test tube after the chloroform layer was pipetted out. Pink colour changes in the resultant solution were detected, which confirmed the presence of anthraquinones (Harborne, 1973).

3.6.1.6 Test for steroids

Crude extract of 0.5 g was mixed with 2 mL 98% H_2SO_4 and 2 mL of acetic anhydride. The presence of steroids was detected by a change in colour from violet to blue (Harborne, 1973).

3.6.1.7 Test for glycosides

One millilitre (1 mL) of acetic acid was added to 0.25 g crude extract of plant material. One drop of 0.1% ferric chloride was added to the mixture, as well as 1 mL of H_2SO_4 (98%). A positive test for glycosides was shown by the production of brown-ring precipitation (Trease & Evans, 1989).

3.6.1.8 Test for tannins

In the test for tannins, 0.125 g crude extract was added to 2.5 mL of dH₂O and the mixture was boiled and filtered while hot. A few drops of 0.1% ferric chloride were added. The formation of a brownish-green or a blue-black colouration was taken as an indication of tannins (Sofowora, 1993).

3.6.2 Quantitative analysis of secondary metabolites

To determine the amount of phytochemicals present in the aqueous and methanol extracts of *M. parviflora*, *E. elephantina*, *P. prunelloides*, and *D. anomala*, the total content of phytochemicals was determined. Spectrophotometric methods adopted from Siddiqui et al. (2017) were used to obtain the results.

3.6.2.1 Total flavonoid content determination

The aluminium chloride colorimetric method was used for flavonoid determination.

(a) Preparation of standard quercetin solution

Standard stock solution (primary) was prepared by dissolving 5.0 mg of quercetin in 1.0 mL of methanol.

(b) Preparation of calibration standard solutions

The calibration standard solutions were prepared by diluting primary stock solution with methanol to get calibration standard solutions of 25, 75, 125, 150, 175, and 200 µL/mL.

(c) Procedure for the estimation of total flavonoid content

An amount of 0.6 mL diluted standard quercetin solutions and extracts were separately mixed with 0.6 mL of 2% aluminium chloride. After mixing, the solutions were incubated for 60 minutes at room temperature. The absorbance of the reaction mixtures was measured against a blank at 420 nm wavelength with a Varian Cary® 50 ultraviolet-visible (UV-Vis) spectrophotometer. The concentration of the total flavonoid content in the test samples was calculated from the calibration plot ($Y = 0.0162X + 0.0044$, $R^2 = 0.999$) and expressed as the milligram quercetin equivalent (QE)/G of plant material. All the determinations were carried out in triplicate.

3.6.2.2 *Total phenolic content determination*

The total phenolic content was determined using the Folin-Ciocalteu assay (Siddiqui et al., 2017).

(a) *Preparation of standard gallic acid solution*

Standard stock solution (primary) was prepared as observed from Siddiqui et al. (2017) by dissolving 100 mg of gallic acid in 10 mL of 80% methanol and then increasing the volume to 100 mL with dH₂O in a 100-mL volumetric flask to obtain a concentration of 1 mg/mL (1 000 µg/mL).

(b) *Preparation of calibration standard solutions*

The calibration standard solutions were prepared by diluting primary stock solution with dH₂O to get calibration standard solutions of 50 µL/mL, 100 µL/mL, 150 µL/mL, 250 µL/mL, and 500 µL/mL of gallic acid solution, and the absorbance was measured at maximum 720 nm against reagent blank.

(c) *Procedure for the estimation of total phenolic content*

One hundred microlitres (100 µL) of each extract solution prepared for a final concentration of 50 to 500 µL/mL, 100 µL of 50% Folin-Ciocalteu reagent, and 2 mL of 7.5% of sodium carbonate were prepared in triplicate. The mixture was incubated for 30 minutes at room temperature and the absorbance was measured at a wavelength of 720 nm.

Gallic acid was used to formulate a standard curve (0 to 350 µg/mL, $Y = 0.0029X - 0.0121$, $R^2 = 0.995$) and the total phenolic content of gallic acid equivalent (GAE) extracts was calculated using the following formula:

$$C = c.V/m$$

Where: C is the total amount of phenolic compounds in mg/g of plant extract in GAE;
c is the concentration of gallic acid from the calibration curve (µg/mg);
V is the extract volume (mL); and
m is the weight of pure organic plant extraction (g).

3.7 ANTIMICROBIAL ASSAY

The disc diffusion assay was employed to screen for the antimicrobial activity of the extracts against selected bacterial species commonly found in wounds.

3.7.1 Disc diffusion assay

3.7.1.1 Microorganisms

All microorganisms were obtained from Pathcare Welkom. The bacterial species used were gram-positive *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228), as well as gram-negative *P. aeruginosa* (ATCC 27853). The fungal species used was *C. albicans* (ATCC 14053). The organisms were maintained on Mueller-Hinton (MH) agar (Merck, South Africa) at 4 °C and cultured in MH broth at 37 °C for 24 hours before the commencement of the bioassays.

(a) Culture and media preparation

Fresh 24-hour cultures were grown for the assay, after which a stock inoculum of each test microbe was prepared and adjusted to a 0.5 McFarland's standard (equivalent to 5×10^5 colony-forming units [CFU]/mL) using a spectrophotometer.

(b) Determination of antimicrobial activity

The disc diffusion assay, as performed by Parker et al. (2017) and Mailafiya (2018), with modifications, was used to determine the inhibitory effect of the extracts on each organism. All tests were conducted following standards set by the Clinical and Laboratory Standards Institute (2009). Serial dilutions with concentrations of 400 µl/mL, 200 µl/mL, 100 µl/mL, and 50 µl/mL were separately prepared for each plant extract. Sterile 6-mm filter paper discs were dipped into each prepared extract so as to impregnate the disc with the respective extract. The discs were dried overnight in a laminar flow cabinet to allow for the evaporation of the solvent. Discs impregnated with 5 µg penicillin for *S. aureus*, *P. aeruginosa*, *S. epidermidis*, and 5 µg fluconazole for *C. albicans* were included as positive controls. Negative control discs were prepared using 1% dimethyl sulfoxide (DMSO) for organic extracts, and sterile dH₂O for the aqueous extracts. The inoculum (100 µL, 5×10^5 CFU/mL) was streaked across the surface of the MH agar plate and spread evenly across the surface of the agar. Zones of inhibition were determined by gently placing the paper discs containing the

plant extracts and controls on the inoculated petri dish and incubating for 24 hours at 37 °C. A calliper was used to measure the diameter of zones of inhibition and expressed as the mean diameter of the zone of inhibition (in mm) of the triplicate measurements.

3.8 CYTOTOXICITY AND CELL PROLIFERATION ASSAY

3.8.1 *Plant stock preparation*

Fifty milligrams (50 mg) of the methanol and aqueous crude extracts of *M. parviflora*, *P. prunelloides*, *D. anomala*, and *E. elephantina* were solubilized using DMSO to make a stock of 100 mg/mL.

3.8.1.1 *Cell culture conditions and maintenance*

All cell culture components and operations were utilised and performed in a sterile and aseptic Class II Biosafety Cabinet.

3.8.1.2 *Human epidermal keratinocyte (HaCaT) cell incubation*

The HaCaT cell lines used in this study were procured from Cellonex and cultured in a humidified (5% carbon dioxide [CO₂]) incubator at 37 °C in low-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% melphalan in a cell culture plate. At 80% confluence, the cells were trypsinised using a 0.25% trypsin / ethylenediaminetetraacetic acid buffer. The growth medium was aspirated from the petri dish, whereafter 5 mL of Dulbecco's phosphate-buffered saline (DPBS) was added to the monolayer culture; gently rocking the cell culture plate to rinse the cells, after which they were aspirated. The rinse was repeated with another 5 mL of DPBS and incubated at 37 °C for five minutes, after which the second DPBS was aspirated. One millilitre (1 mL) of trypsin was added to the cells and the cell culture plate was rocked sideways to ensure that all cells were covered thoroughly, and then aspirated. The plate was then incubated for approximately 10 minutes at 37 °C in a 5% CO₂ incubator. To ensure that the cells were detached, they were observed under a microscope. One millilitre (1 mL) of complete medium was added to the plate and the medium was then pipetted up and down five to 10 times to dislodge and re-suspend the cells.

3.8.1.3 Cell viability test

Cell viability and numbers were routinely monitored using a haemocytometer slide and the trypan blue assay. Trypan blue is an acidic dye with a molecular weight of approximately 873 g/mol. It is a large, hydrophilic, tetra-sulphonated molecule and contains two azo chromophores. Due to its negative charge, trypan blue is unable to enter the cytoplasm of viable cells whose membranes are intact. Non-viable cells, however, have compromised cell membranes and trypan blue is thus able to enter these cells where it can bind to intracellular proteins within the cytoplasm of the cell effectively staining the cytoplasm blue. The blue colour is readily observed under a brightfield microscope thus allowing the easy and rapid differentiation between live (clear cytoplasm) and non-viable (blue cytoplasm) cells (Pringle, 2017). Trypan blue stain was prepared freshly as a 0.4% solution in 0.9% sodium chloride before each experiment was started. Cells in 96 wells were washed in PBS and dispersed with 0.025% trypsin- EDTA. After trypsinization, 10 μ L cell suspension was added to 10 μ L of trypan blue solution and mixed thoroughly. The mixture was allowed to stay at room temperature for 3 minutes. Immediately following the incubation period of 3 minutes, a 10 μ L mixture of the trypan blue/cell mixture was added to a hemacytometer, which is placed on the stage of a binocular microscope. Immediately after the incubation time. Triplicate wells of viable cells were counted using a hemocytometer and the experiment was repeated three times. Cell viability was expressed as the percentage of cells staining blue.

3.8.1.4 Evaluation of cytotoxicity and cell proliferation (in vitro)

Hoechst 33342 and propidium iodide (PI) dual staining were used in conjunction with an ImageXpress Micro XLS Widefield microscope (Molecular Devices®) for high-content analysis to screen for the cytotoxicity of the plant crude extracts. Any plant crude extract that was found to be cytotoxic was eliminated from further testing.

HaCaT cells (5×10^3 cells/wells in a 96-well plate) from the plate were seeded in the growth medium/well and incubated at 37 °C in 5% CO₂. After 24 hours of incubation, the supernatant was discarded and the cells were pre-treated with a growth medium, followed by mixing with different concentrations of methanol and aqueous extracts of *M. parviflora*, *E. elephantina*, *P. prunelloides*, and *D. anomala* at 50 and 200 μ g/mL in

triplicate and incubated for a period of 24 hours and 48 hours for proliferation screening respectively. The medium was gently removed by pipette, and the cells were incubated in 50 μL of Hoechst 33342 (5 $\mu\text{g}/\text{mL}$ in Phosphate buffered saline PBS + $\text{Ca}^{2+}/\text{Mg}^{2+}$) for 10 minutes at room temperature and protected from light. Immediately before acquiring the images, 50 μL of PI (100 $\mu\text{g}/\text{mL}$ in PBS + $\text{Ca}^{2+}/\text{Mg}^{2+}$) was added. Culture medium and melphalan were used as controls. Nine image locations encompassing approximately 80% of the well were collected using a 10x magnification. MetaXpress® software was used to compute the average cell count.

3.9 ANTIOXIDANT ACTIVITY ASSAY

3.9.1 Nitric oxide (NO) scavenging assay

ROS assay using tert-Butyl hydroperoxide (TBHP) and CellROX Orange were used to test the efficacy of the aqueous extracts (*P. prunelloides*, *E. elephantina*, *M. parviflora* whole plant, and *M. parviflora* leaves) to protect the HaCaT cells from oxidative stress (Thermo Fisher Scientific). The HaCaT cells (5×10^3 cells/well in 96-well plates) were seeded overnight and pre-treated with concentrations of 50 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$ of the aqueous extracts for 48 hours at 37 °C. Thereafter, oxidative stress was generated by adding 100 μM TBHP to the media for two hours at 37 °C, as described by Toussaint et al. (2002), with some changes. Catechin was included as a positive control. The cells were stained with CellROX Orange (5 μL) and Hoechst 33342 (5 $\mu\text{g}/\text{mL}$ in PBS) as directed by the manufacturer for 30 minutes at 37 °C, and covered from light, after which the absorbance was measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, Vermont, USA) and fluorescence micrographs were taken.

The percentage of NO scavenged was calculated using the following formula:

$$\% \text{ NO scavenging} = A - B / A \times 100$$

Where: A is the absorbance value of the positive control; and
B is the absorbance value of the test sample.

3.10 ANTI-INFLAMMATORY ASSAY

RAW 264.7 mouse macrophages were purchased from Cellonex (South Africa). Lipopolysaccharide (LPS), Griess reagent, and aminoguanidine were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). RPMI (Roswell Park Memorial Institute) 1640 culture medium and FBS were obtained from GE Healthcare Life Sciences (Logan, Utah, USA).

3.10.1 Sample preparation

Five aqueous extracts were solubilised using 50 μ L of DMSO to make a stock of 100 mg/mL. Extracts were stored at 4 °C until used. Aminoguanidine (100 μ L) was used as a positive control to indicate anti-inflammatory activity.

3.10.2 Anti-inflammatory screening

RAW 264.7 cells were seeded in RPMI1640 culture medium supplemented with 10% FBS (RPMI complete medium) into 96-well plates at a density of 1×10^5 cells per well and allowed to attach overnight. The following day, the culture medium was removed, and the samples were added to give final concentrations of 50, 100, and 200 μ g/mL. To assess the anti-inflammatory activity, 50 μ L of LPS (final concentration of 500 μ g/mL) containing medium was added to the corresponding wells. Aminoguanidine was used as the positive control at 100 μ L. The cells were incubated further for 24 hours. To quantify NO production, 50 μ L of the spent culture medium was transferred to a new 96-well plate, and 50 μ L of Griess reagent was added. Absorbance was measured at 540 nm. A standard curve using sodium nitrate dissolved in a culture medium was used to determine the concentration of NO in each sample.

3.10.3 Cytotoxicity screening protocol

MTT was used to measure cell viability in order to verify that toxicity was not a contributing factor. In order to achieve this, the residual medium and treatments in each well were removed, and new medium containing 0.5 mg/mL MTT was added. The mixture was then further incubated for 30 minutes at 37°C. Subsequently, 100 μ L DMSO was applied to each well to dissolve the formazan crystals after the removal of MTT. Absorbance was measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

3.11 TUBE FORMATION ASSAY

Plastic culture dishes (60 mm) were pre-coated with 2 g of collagen in 12 mL of warm dH₂O overnight before the HeLa cells were prepared. Thereafter, a collagen solution of 2 mL was administered into each of the six-well plates and placed in a sterile hood overnight. Immediately before use, 2 mL of fresh medium was added to each well to neutralise the collagen and put in a -20 °C freezer for one hour to obtain ice-cold neutralised collagen. Approximately 0.5 mL of the plant extract of *E. elephantina* was layered onto the collagen, ensuring that the bottom was completely covered. Freshly coated dishes were placed at 37 °C in a humidified incubator for one hour to allow the matrix material to gel. Following that, 100 µL of matrigel was added to each well and incubated at 37 °C for 30 minutes. The HeLa cells were then seeded at 20.000 cells/mL density (~1 000 cells/well). After attachment (12 hours at 37 °C), the cells were covered with a second layer of matrigel (sandwich), which was allowed to polymerise overnight at 37 °C. The following day, the cells were observed via microscope (20x) to ensure a matrigel sandwich of cell and then cultured for three days. After the three days, the dish was assessed again via microscope (20x) to observe network formation and the tubular-like structure.

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 INTRODUCTION

This chapter reports on the results obtained from the study and concisely describes what was observed and measured during the study. In this chapter, the results obtained from the wound-healing and antimicrobial bioassays and phytochemical screening as mentioned in the methodology chapter are presented and supported by tables and graphs. The discussions follow respectively.

4.2 PHYTOCHEMICAL SCREENING

For many decades, phytochemicals have been recognised for their therapeutic properties, mostly due to their antioxidant, anti-inflammatory, anti-proliferative, and anti-carcinogenic qualities (Anand et al., 2017). High concentrations of phytochemicals such as flavonoids, alkaloids, tannins, anthraquinones, phenols, and saponins were observed in the tested plants. These secondary metabolites have been reported to contribute towards wound healing or skin treatment (Vitale et al., 2022).

Table 4.1 presents the qualitative results of the phytochemical constituents found in *P. prunelloides*, *E. elephantina*, *M. parviflora* whole plant, *M. parviflora* leaves, and *D. anomala*.

Table 4.1: Qualitative analysis of phytochemical constituents found in *P. prunelloides*, *E. elephantina*, *M. parviflora* whole plant, *M. parviflora* leaves, and *D. anomala*

Compound	Species	Methanol extract	Aqueous extract
Alkaloids	<i>D. anomala</i>	-	-
	<i>E. elephantina</i>	-	-
	<i>P. prunelloides</i>	+	-
	<i>M. parviflora</i>	+	+
Anthraquinones	<i>D. anomala</i>	-	-
	<i>E. elephantina</i>	+	+
	<i>P. prunelloides</i>	+	+
	<i>M. parviflora</i>	+	+
Flavonoids	<i>D. anomala</i>	+	+
	<i>E. elephantina</i>	+	-
	<i>P. prunelloides</i>	-	+
	<i>M. parviflora</i>	+	+
Glycosides	<i>D. anomala</i>	-	-

Compound	Species	Methanol extract	Aqueous extract
	<i>E. elephantina</i>	+	+
	<i>P. prunelloides</i>	+	+
	<i>M. parviflora</i>	+	+
Saponins	<i>D. anomala</i>	+	-
	<i>E. elephantina</i>	+	+
	<i>P. prunelloides</i>	+	+
	<i>M. parviflora</i>	-	+
Steroids	<i>D. anomala</i>	+	+
	<i>E. elephantina</i>	+	-
	<i>P. prunelloides</i>	-	+
	<i>M. parviflora</i>	+	+
Tannins	<i>D. anomala</i>	+	+
	<i>E. elephantina</i>	+	+
	<i>P. prunelloides</i>	+	+
	<i>M. parviflora</i>	-	+
Terpenoids	<i>D. anomala</i>	+	+
	<i>E. elephantina</i>	+	-
	<i>P. prunelloides</i>	-	+
	<i>M. parviflora</i>	-	-
Phenols	<i>D. anomala</i>	+	+
	<i>E. elephantina</i>	+	+
	<i>P. prunelloides</i>	-	-
	<i>M. parviflora</i>	+	+

Key: The qualitative results were reported as (+) if phytochemicals were present and (-) if absent.

The *M. parviflora* methanol and aqueous extracts showed the presence of flavonoids, alkaloids, anthraquinones, glycosides, steroids, and phenols, whereas saponins and tannins were detected only in the aqueous extracts. The findings on these phytochemical constituents in *M. parviflora* reported in this study concur with those of Farhan et al. (2012).

The aqueous extract of *P. prunelloides* displayed the presence of flavonoids and steroids, while the methanol extract displayed only the presence of alkaloids. However, both extracts had glycosides, saponins, tannins, and anthraquinones. These respective phytochemicals were previously identified by Mpofu et al. (2014) and Ndlovu (2009).

Tannins, saponins, glycosides, anthraquinones, and phenols were detected in the aqueous and methanol extracts of *E. elephantina*. The methanol extract further showed the presence of flavonoids, steroids, and terpenoids, which were not detected in the aqueous extract.

The results of both the *D. anomala* aqueous and methanol extracts showed the presence of terpenoids, steroids, flavonoids, and phenols, while saponins were only detected in the methanol extract.

The total phenolic and flavonoid contents of the 10 extracts from four medicinal plants were analysed (see Table 4.2). Gallic acid and quercetin curves were used as standards. On the basis of their regression lines, the equivalents of the standards were determined as milligram of gallic acid (mg GAE/g) or (QE)/G of dry sample.

Table 4.2: Quantitative estimate of phytochemicals present in *P. prunelloides*, *E. elephantina*, *M. parviflora* whole plant, *M. parviflora* leaves, and *D. anomala*

Species name	Extract type	Total flavonoid content mg QE/g (mean $\pm$ SD)	Total phenol content mg GAE/g (mean $\pm$ SD)
<i>D. anomala</i> (roots)	Methanol	127 $\pm$ 1,1	29 $\pm$ 0,07
	Aqueous	62 $\pm$ 0,50	13 $\pm$ 0,05
<i>P. prunelloides</i> (roots)	Methanol	171 $\pm$ 1,5	32 $\pm$ 0,07
	Aqueous	264 $\pm$ 2,4	45 $\pm$ 0,09
<i>E. elephantina</i> (roots)	Methanol	Not tested	Not tested
	Aqueous	140 $\pm$ 0,19	134 $\pm$ 0,19
<i>M. parviflora</i> (whole plant)	Methanol	147 $\pm$ 1,3	32 $\pm$ 0,07
	Aqueous	309 $\pm$ 2,8	16 $\pm$ 0,05
<i>M. parviflora</i> (leaves)	Methanol	Not tested	Not tested
	Aqueous	397 $\pm$ 3,6	Not tested

SD = Standard deviation

The *M. parviflora* whole plant and leaf aqueous extracts showed the highest total flavonoid content, 397 and 309 mg GAE/g respectively, followed by the *P. prunelloides* aqueous extract, which had a value of 264 mg GAE/g. The tested methanol extracts of *P. prunelloides*, *M. parviflora* leaves, and *D. anomala* were more or less in the same range for the total flavonoid content. There was a generally low total phenolic content for the tested methanol and aqueous extracts in this study compared to total flavonoid content. However, *E. elephantina* was the only plant that showed a very high total phenolic content of 134 mg GAE/g. Samples not tested were due to the unavailability of the extracted sample.

Although the total phenolic content for the *M. parviflora* leaf aqueous extract was not tested in this study due to the unavailability of the extract sample, Farhan et al. (2012) demonstrated that the total phenolic content was 40 mg/mL for the *M. parviflora* leaf aqueous extract. In addition, Rahab et al. (2019) found that the methanol extract of *M. parviflora* leaves had a total phenolic content of 390.7 mg GAE/g, while the total flavonoid amount was 203.7 mg GAE/g. A study by Mazhangara et al. (2020) revealed

that the quantitative phytochemical analysis of *E. elephantina* methanol extract showed a total flavonoid content of 803.93 mg GAE/g and a total phenolic content of 476.11 mg GAE/g.

Flavonoids, alkaloids, tannins, anthraquinones, phenols, and saponins are responsible for the removal of free radicals, increasing the contraction of the wound area, and for increasing the formation of blood vessels and fibroblasts (Hosseinkhani et al., 2017). As such, *D. anomala*, *E. elephantina*, *P. prunelloides*, and *M. parviflora* could have good wound-healing potential because they possess different phytochemicals such as flavonoids, alkaloids, tannins, anthraquinones, phenols, and saponins. Phytochemicals have been found to possess the ability to act as anti-inflammatory agents that are capable of modifying the activities of inflammatory cells and are thus considered as wound-healing agents (Iwalewa et al., 2007).

Phytochemicals promote the wound-healing process by increasing the rate of epithelialisation, tensile strength, and hydroxyproline content (Ghosh & Gaba, 2013; Mittal et al., 2013; Tsala et al., 2013; Pawar & Toppo, 2015). Additionally, flavonoids, alkaloids, tannins, phenols, and saponins play a protective role in defending the body from damage caused by a variety of stressors by, among others, absorbing UV radiation and acting as anti-proliferative agents (Kennedy & Wightman, 2011; Saxena et al., 2013).

The *M. parviflora* methanol and aqueous extracts showed the presence of flavonoids, alkaloids, phenols, saponins, and tannins, which can be attributed to antimicrobial and wound-healing properties by stimulating the fibroblasts and vascular endothelial cells, as well as their regulation to alter the cellular mechanisms. These actions by these phytochemicals decrease the likelihood of microbial infection (Schultz et al., 2003; Borges et al., 2016) and thus make *M. parviflora* a potential wound-healing agent. Anthraquinones, glycosides, and steroids were also detected in the methanol and aqueous extracts of *M. parviflora* and they may play a role in the removal of free radicals, increasing the contraction of the wound area, and increasing the formation of blood vessels and fibroblasts (Hosseinkhani et al., 2017).

P. prunelloides displayed the presence of flavonoids and steroids in the aqueous extract, while the methanol extract displayed the presence of alkaloids. However, both extracts were found to contain glycosides, saponins, tannins, and anthraquinones.

These results reveal that *P. prunelloides* has wound-healing abilities by inhibiting microbial infection through its antimicrobial activity (Schultz et al., 2003), by being an antioxidant (Hosseinkhani et al., 2017), and by possessing anti-inflammatory properties (Saxena et al., 2013) through its phytochemical constituents. Tannins, saponins, glycosides, anthraquinones, and phenols were detected in the aqueous and methanol extracts of *E. elephantina*. *E. elephantina* is therefore suitable as an anti-inflammatory agent that promotes capillary vasoconstriction, which in turn reduces vascular permeability and thus promoting wound healing (Saxena et al., 2013).

The *D. anomala* aqueous and methanol extracts showed the presence of terpenoids, steroids, flavonoids, and phenols. The results suggest that *D. anomala* has wound-healing potential by playing an important role in scavenging free radicals and fighting infection; thus, promoting faster wound healing (Rajkumar et al., 2018).

4.3 ANTIMICROBIAL SCREENING

Wounds provide a moist, warm, and nutritive environment that is optimal for microbial growth, and the infection of wounds by microorganisms can hamper healing (Houghton et al., 2005). The antimicrobial properties of medicinal plants are thus also determined when validating wound-healing properties. Microflora that have been isolated from wounds and used in in vitro assays include *S. aureus*, *P. aeruginosa*, *S. epidermidis*, and *C. albicans* (Egbe et al., 2011), which were screened in this study to investigate the antimicrobial properties of *P. prunelloides*, *M. parviflora*, *D. anomala*, and *E. elephantina*. The antibacterial and antifungal activities of aqueous extracts prepared from the four plants under study were investigated using the agar disc diffusion method. Methanol extracts were not tested because they had shown to be cytotoxic during the cytotoxicity experiment and were therefore excluded from this assay.

The results for antimicrobial activity are presented in Table 4.3. The *E. elephantina* aqueous extract at a minimum inhibition concentration (MIC) of 50 µg/mL displayed the most promising antifungal activity, with a minimum zone of inhibition (MZI) of 12.4 mm against *C. albicans*. This was followed by the *P. prunelloides* aqueous extract, with an MIC of 100 µg/mL, which also exhibited a slightly higher MZI of 13.8 mm against *C. albicans* than the other medicinal plants tested. These findings

lend credence to studies by Xaba (2016) and Machaba and Mahlo (2017). *M. parviflora*, on the other hand, did not show any signs of antifungal activity against the *C. albicans* strain and had a zone of inhibition with a diameter of 6 mm. This is surprising because *M. parviflora* has been reported to exhibit some antifungal properties by Islam et al. (2010) and Navneet (2017). This could be because of the solvent type that was used in this study as previous studies reported on organic extracts instead of aqueous extracts (Islam et al., 2010; Navneet, 2017). These findings of low and no activity against antifungal strains may be because it is more difficult to extract the components that are responsible for the action of the antimicrobial agent in aqueous extracts than in organic extracts (Machaba & Mahlo, 2017). According to Machaba and Mahlo (2017), phytochemicals with antimicrobial activity are not easily extracted with water as they are hydrophobic; they are extracted better with organic solvents.

The *M. parviflora* leaf aqueous extract showed antibacterial activity at the concentration of 100 µg/mL with the zone of inhibition diameter of 20 mm, 22 mm, and 18 mm for *S. epidermidis*, *S. aureus*, and *P. aeruginosa* respectively. Moreover, the aqueous extract prepared from *M. parviflora* (whole plant) also exhibited excellent antibacterial activity against these three bacteria with a zone of inhibition ranging from 25 to 28 mm at the MIC of 50 µg/mL to 100 µg/mL (see Table 4.3). This may be the result of the presence of very high flavonoid and phenolic content in the aqueous extracts.

P. prunelloides showed good antibacterial activity against *S. epidermidis* (15 mm), *S. aureus* (15 mm), and *P. aeruginosa* (19 mm). Interestingly, the MIC across the three bacteria was the same at 50 µg/mL even though the zone of inhibition differed. This good antibacterial activity can be attributed to the phytochemicals that were detected in the extracts, such as glycosides, saponins, tannins, and anthraquinones, as discussed in the phytochemical results section.

E. elephantina also showed good antibacterial activity across the three tested bacteria with the zone of inhibition at 17 mm, 15 mm and 15 mm for *S. epidermidis* (MIC = 100 µg/mL), *S. aureus* (MIC = 100 µg/mL), and *P. aeruginosa* (MIC = 50 µg/mL) respectively. *E. elephantina* also exhibited the highest phenolic content, which could be a contributing factor to the antibacterial results.

The *D. anomala* results showed varying effectiveness against the three bacteria tested. *S. epidermidis* had the highest MIC of 200 µg/mL, followed by *S. aureus* at 100 µg/mL, and *P. aeruginosa* at 50 µg/mL. The zones of inhibition also varied. They were observed as 20 mm for *S. epidermidis*, 17 mm for *S. aureus*, and 18 mm for *P. aeruginosa*. The reason behind the increased effectiveness against gram-positive bacteria when compared to gram-negative bacteria is because gram-positive bacteria have a more permeable cell wall (Xaba, 2016). The gram-positive organisms tested in this study were found to be vulnerable since they only have a thin coating of peptidoglycan that serves as a permeability barrier, which makes them easily accessible to the different plant extracts they were tested against. In comparison to gram-positive bacteria, gram-negative organisms have more complex cell walls that operate as a diffusional barrier, which makes them more resistant to antimicrobial drugs (Xaba, 2016).



Table 4.3: Zone of inhibition of extracts (mm) against different species or bacteria using the agar disc diffusion method

Plant extract	Concentration (µg/mL)	<i>C. albicans</i>	<i>S. epidermidis</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
<i>E. elephantina</i>	400	++	++	++	++
	200	++	++	++	++
	100	++	++	++	++
	50	++	-	-	++
	+ control	++	++	++	++
	- control	-	-	-	-
		MIC = 50 µg/mL MZI = 13 mm	MIC = 100 µg/mL MZI = 17 mm	MIC = 100 µg/mL MZI = 15 mm	MIC = 50 µg/mL MZI = 15 mm
<i>D. anomala</i>	400	-	++	++	++
	200	-	++	++	++
	100	-	-	++	++
	50	-	-	-	++
	+ control	++	++	++	++
	-control	-	-	-	-
		MIC = N/A	MIC = 200 µg/mL MZI = 20 mm	MIC = 100 µg/mL MZI = 17 mm	MIC = 50 µg/mL MZI = 18 mm
<i>P. prunelloides</i>	400	-	++	++	++
	200	-	++	++	++
	100	-	++	++	++
	50	-	++	++	++
	+ control	+	+	+	+
	- control	-	-	-	-
		MIC = N/A	MIC = 50 µg/mL MZI = 15 mm	MIC = 50 µg/mL MZI = 15 mm	MIC = 50 µg/mL MZI = 19 mm



Plant extract	Concentration (µg/mL)	<i>C. albicans</i>	<i>S. epidermidis</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
<i>M. parviflora</i> (leaves)	400	-	++	++	++
	200	-	++	++	++
	100	-	++	++	++
	50	-	-	-	-
	+ control	++	++	++	++
	- control	-	-	-	-
		MIC = N/A	MIC = 100 µg/mL MZI = 20 mm	MIC = 100 µg/mL MZI = 22 mm	MIC = 100 µg/mL MZI = 18 mm
<i>M. parviflora</i>	400	-	++	++	++
	200	-	++	++	++
	100	-	++	++	++
	50	-	-	-	-
	+ control	++	++	++	++
	- control	-	-	-	-
		MIC = N/A	MIC = 100 µg/mL MZI = 25 mm	MIC = 100 µg/mL MZI = 27 mm	MIC = 50 µg/mL MZI = 28 mm

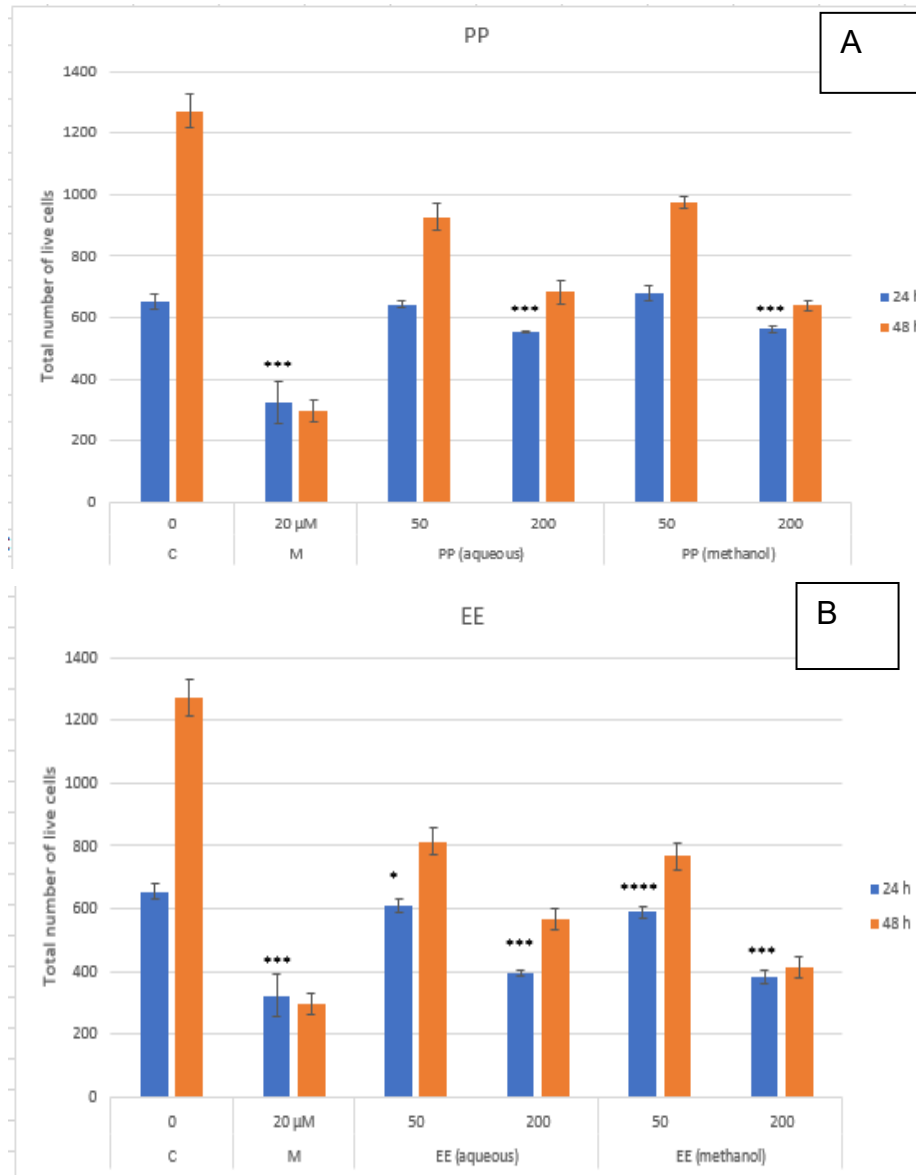
Key: N/A = Not active; MIC (mg/mL); ++ = Sensitive, where zone of inhibition is >16 mm; - = Resistance, where zone of inhibition is <16 mm

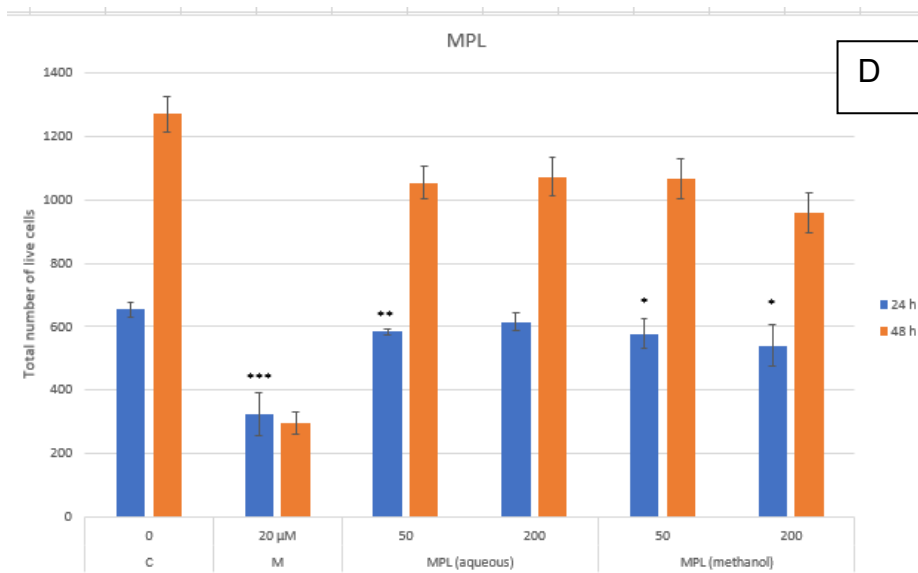
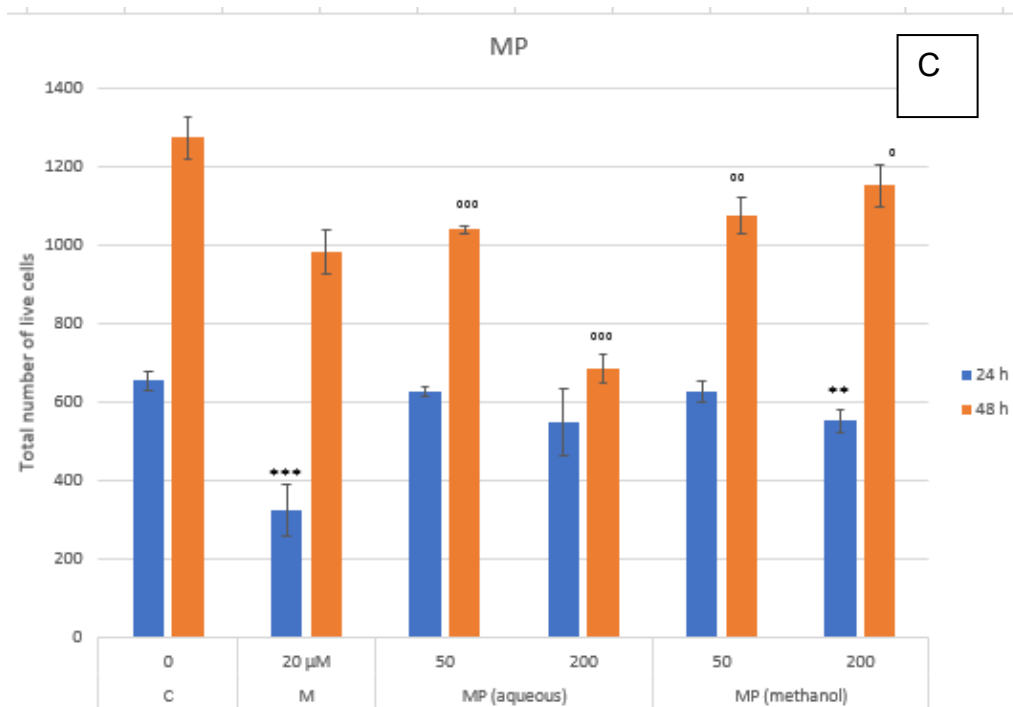
4.4 CELLULAR PROLIFERATION ASSAY

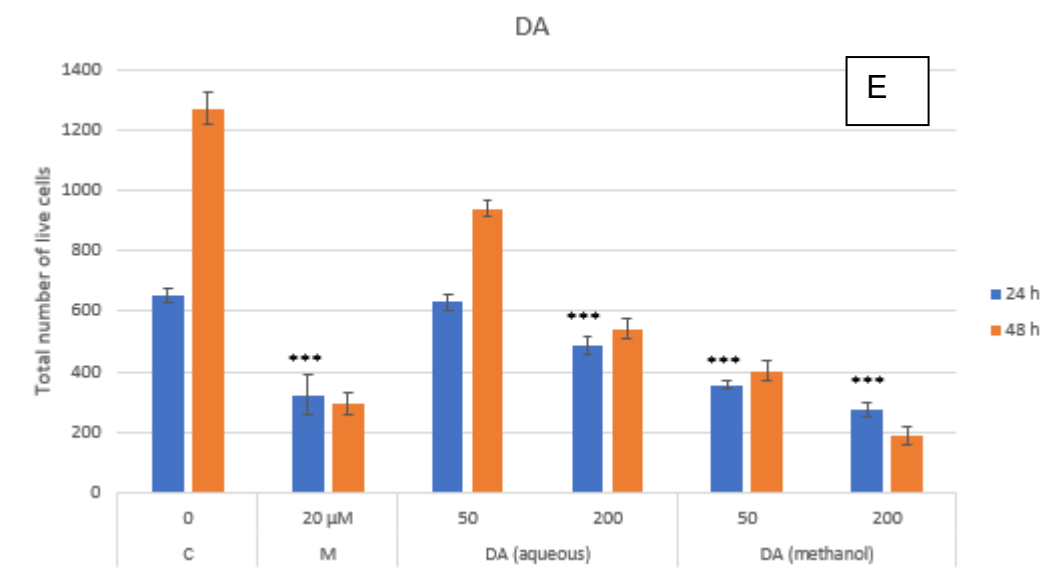
4.4.1 *Screening of extracts for potential wound-healing properties through the determination of their effect on cellular proliferation*

Cellular proliferation testing was conducted to guarantee that the selected concentrations of each of the plant crude extracts did not cause substantial cell death to the HaCaT cells. In addition, the antioxidant, anti-inflammatory, and tubular formation capability of each plant crude extract was evaluated to determine the potential advantages of each extract in countering oxidative stress and increased wound-healing process. Cytotoxicity screening of the aqueous and methanol extracts of *P. prunelloides*, *E. elephantina*, *M. parviflora* whole plant, *M. parviflora* leaves, and *D. anomala* on HaCaT cells was carried out using Hoechst 33342 and propidium iodide (PI) dual staining in conjunction with Molecular Devices ImageXpress Micro XLS Widefield microscope for high content analysis.

Figure 4.1A to 4.1E depicts the findings of the cytotoxicity screening tests on the HaCaT cells for each of the plant crude extracts used in this investigation. The untreated sample consisted of low-glucose DMEM supplemented with 10% FBS and represents 100% cell density, while melphalan was used as a positive control to assess the cytotoxicity of the crude extracts. This means that if the crude extracts of the plants exhibit the same results as melphalan, they are cytotoxic to the HaCaT cells and those are not desired results for this study.







Bar graphs represent triplicate values of one experiment. Error bars represent the SD of the mean.

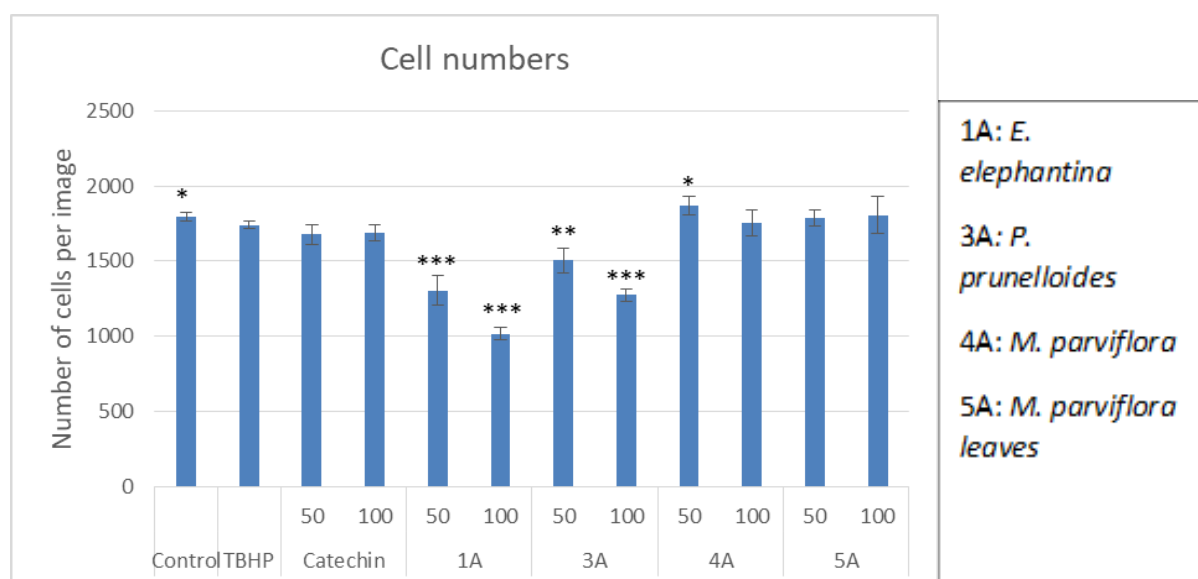
Figure 4.1: Cellular proliferation screening of aqueous and methanol crude extracts of (A) *P. prunelloides*, (B) *E. elephantina*, (C) *M. parviflora* whole plant, (D) *M. parviflora* leaves, and (E) *D. anomala* at two different concentrations (50 and 200 µg/mL) on HaCaT cells after 24 hours and 48 hours with melphalan as a positive control (* $p < 0.05$, ** $p < 0.005$, * $p < 0.001$, **** $p < 0.01$; ° $p < 0.05$, °° $p < 0.005$, °°° $p < 0.001$, °°°° $p < 0.01$ compared to melphalan).**

In general, the *E. elephantina* and *D. anomala* methanol extracts to varying degrees were toxic to HaCaT cells and thus inhibited cell proliferation. As observed in Figure 4.1A, the *P. prunelloides*, showed good cellular proliferation at 200 µg/mL concentration ($p < 0.001$). *M. parviflora* whole plant (figure 4.1C) displayed good cellular proliferation ($p < 0.001$) only after 48 hours for both concentrations (50 and 200 µg/mL) while *M. parviflora* leaf methanol extract and aqueous extract (figure 4.1D) showed good cellular proliferation at both concentrations (50 and 200 µg/mL) and for both time intervals of 24 hours and 48 hours. The aqueous extract for *M. parviflora* leaves indicated only a very small decline in number of approximately 200 cells in comparison to the control group. The *E. elephantina* aqueous extract at low concentration (50 µg/mL) showed negligible cytotoxicity and was considered for further experiments in this study. *D. anomala*, however, showed cytotoxicity even at low concentrations of 50 µg/mL for both the methanol and aqueous extracts; it was thus eliminated from further assays. Chota et al. (2022) also reported the toxic trait of *D. anomala* root methanol extracts after observing their cytotoxicity on MCF-7 cells through induced

cell death that led to decreased cell viability. This was not the case, however, in the in vivo study by Balogun et al. (2016), as they found that the administration of an aqueous root extract of *D. anomala* to Wistar rats was not cytotoxic in all the tested doses and did not reveal any toxicity. The reason for these results obtained by Balogun et al. (2016) could be that the liver enzymes of the Wistar rats metabolised the toxicity of the *D. anomala* extract, which made the extract safer for the body to process (Balogun et al., 2016; Mfengwana, 2022).

4.5 CELLULAR ANTIOXIDANT ASSAY

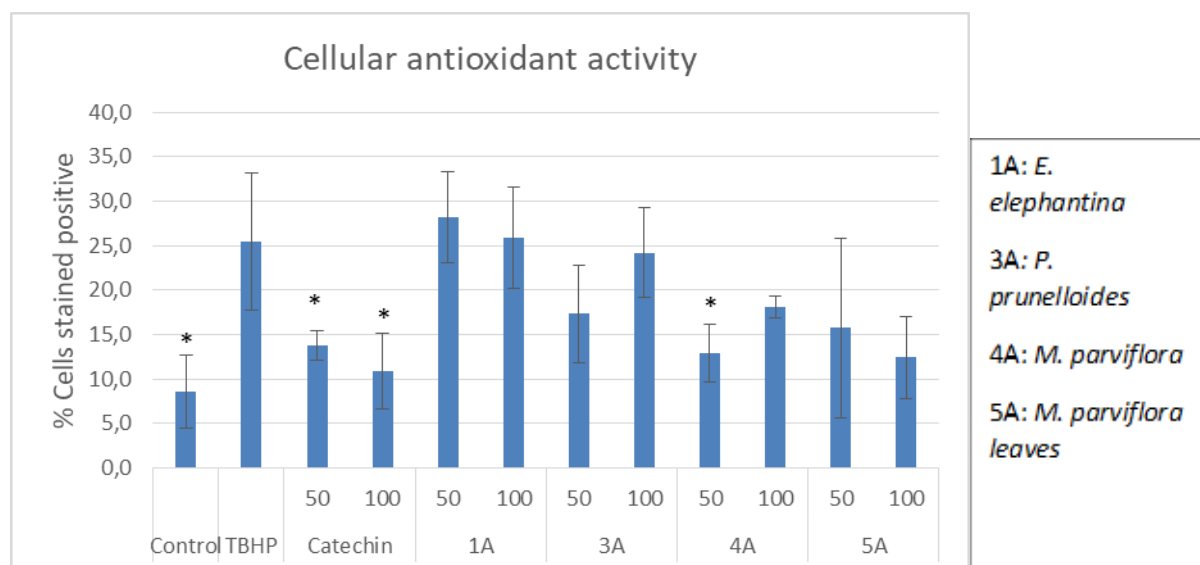
The antioxidant capability of the *E. elephantina*, *P. prunelloides*, *M. parviflora* whole plant, and *M. parviflora* leaf extracts were evaluated using the NO scavenging test since there is a correlation between oxidative stress and poor wound healing (Deng et al., 2021). The HaCaT cells were treated with TBHP to test *E. elephantina*, *P. prunelloides*, *M. parviflora* whole plant, and *M. parviflora* leaf extracts capacity to shield them from oxidative stress. CELLROX was then used to assess the extracts effects on oxidative stress and cell death.



Bar graphs represent triplicate values of one experiment. Error bars represent the SD of the mean.

Figure 4.2: Effects of 48-hour pre-treatment with plant extracts (*E. elephantina*, *P. prunelloides*, *M. parviflora*, and *M. parviflora* leaves) on induced cell death of HaCaT cells after incubation in TBHP (* $p < 0.05$, ** $p < 0.005$, * $p < 0.001$, **** $p < 0.01$, compared to TBHP).**

HaCaT cells were pre-treated with 50-100 µg/mL of the *E. elephantina*, *P. prunelloides*, *M. parviflora*, and *M. parviflora* leaf extracts for 24 hours, followed by exposure to 100 µg/mL TBHP for two hours. Figure 4.2 shows that the cells were still viable following pre-treatment with the 50 and 100 µg/mL concentrations of *E. elephantina*, *P. prunelloides*, *M. parviflora*, and *M. parviflora* leaf extract and subsequent exposure to TBHP. However, the plant extract of *E. elephantina* showed a significant ($p < 0.001$) attenuated number of cells between the two concentrations of 50 and 100 µg/mL, and the *P. prunelloides* plant extract showed a reduced number of cells at a concentration of 100 µg/mL. Cells treated with only TBHP showed no significant death when compared to the negative control group but the *M. parviflora* plant extract at both concentrations of 50 µg/mL and 100 µg/mL significantly attenuated TBHP-induced cytotoxicity. These results show that *M. parviflora* indeed has antioxidant protection due to the direct interaction between the extracts and the ROS, which limits the damage to cell membranes and thus preserves cell life (Süntar et al., 2012).



Bar graphs represent triplicate values of one experiment. Error bars represent the SD of the mean.

Figure 4.3: Effects of *E. elephantina*, *P. prunelloides*, *M. parviflora*, and *M. parviflora* leaf extracts at the maximum non-toxic dose on intracellular ROS levels in HaCaT cells after two hours of incubation in TBHP (* $p < 0.05$, ** $p < 0.005$, * $p < 0.001$, **** $p < 0.01$, compared to TBHP)**

In this study, the degree of ROS accumulation was evaluated in HaCaT cells pre-treated with 50 and 100 µg/mL of *E. elephantina*, *P. prunelloides*, *M. parviflora*, and

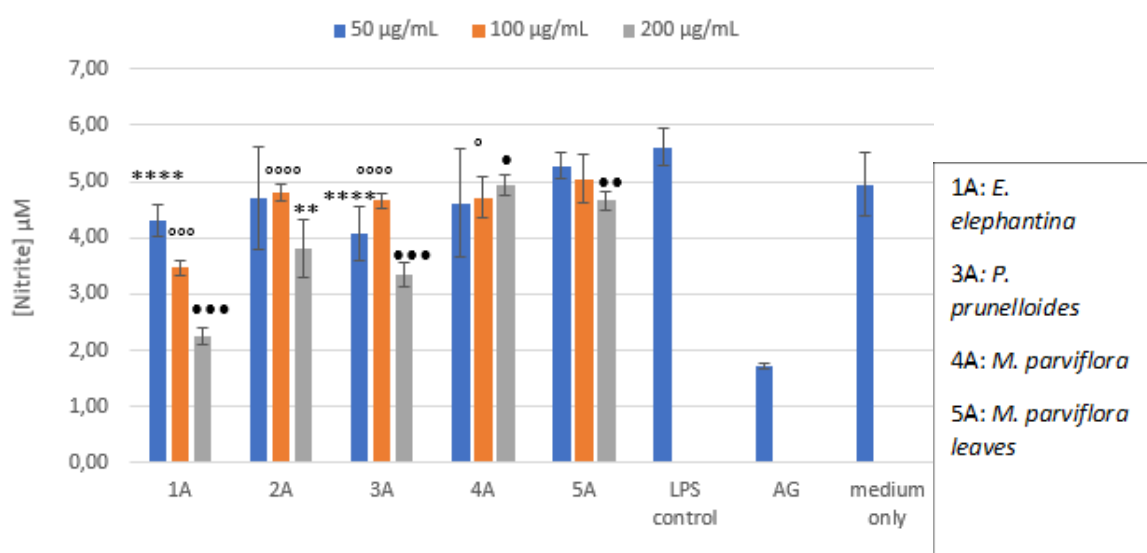
M. parviflora leaf extracts for 24 hours, followed by two hours of incubation in 100 µg/mL of TBHP. As shown in Figure 4.3, the TBHP-only treatment resulted in an increase in cells stained positive with CellROX intensity by 16.9% relative to the control. Treatment with the *E. elephantina* and *P. prunelloides* extract was found to reduce ROS generation relative to the TBHP-only treatment, but the observed reduction was not significant. On the other hand, treatment with the *M. parviflora* extract at a concentration of 50 µg/mL significantly reduced ROS generation by 12.5% relative to the TBHP-only treatment.

Importantly, it was observed that catechin, which is a class of flavonoids and a plant-based chemical that helps to protect plants from environmental toxins and repairs damage, had a strong ability to counteract the oxidant activity of the organic peroxide TBHP by preventing the rise of intracellular ROS. Additionally, the *M. parviflora* and *M. parviflora* leaf extracts also displayed the same effects as the catechin by showing a strong antioxidant effect, as seen in Figure 4.3. This antioxidant potential of the *M. parviflora* and *M. parviflora* leaf extracts can be attributed to the fact that the plant contains flavonoids and phenolic compounds, as shown in Table 4.1. *M. parviflora* also possesses glycosides, tannins, saponins, steroids, alkaloids, and anthraquinones. These bioactive compounds are reported to play a crucial part in the effective treatment and management of wounds due to their ability to reduce the negative effects of ROS by removing products of inflammation and limiting damage to the cells (Süntar et al., 2012). These bioactive compounds are responsible for the elimination of free radicals, the reduction in the size of the wound area, and the acceleration of the development of blood vessels and fibroblasts (Hosseinkhani et al., 2017). *M. parviflora* extract could therefore be a potential source of new drugs for the treatment of skin wounds.

The absence of ROS-accumulation prevention by the *P. prunelloides* plant extract is likely linked to the much higher concentration of total phenols present in this extract, as shown in Table 4.2. Overall, this study confirms the potential of *M. parviflora* extract as a potential wound-healing agent, mainly due to its phytochemical content, non-cytotoxicity on HaCaT cells, and antioxidant properties on skin cells (HaCaT cells) (Mohammed et al., 2022).

4.6 ANTI-INFLAMMATORY ASSAY

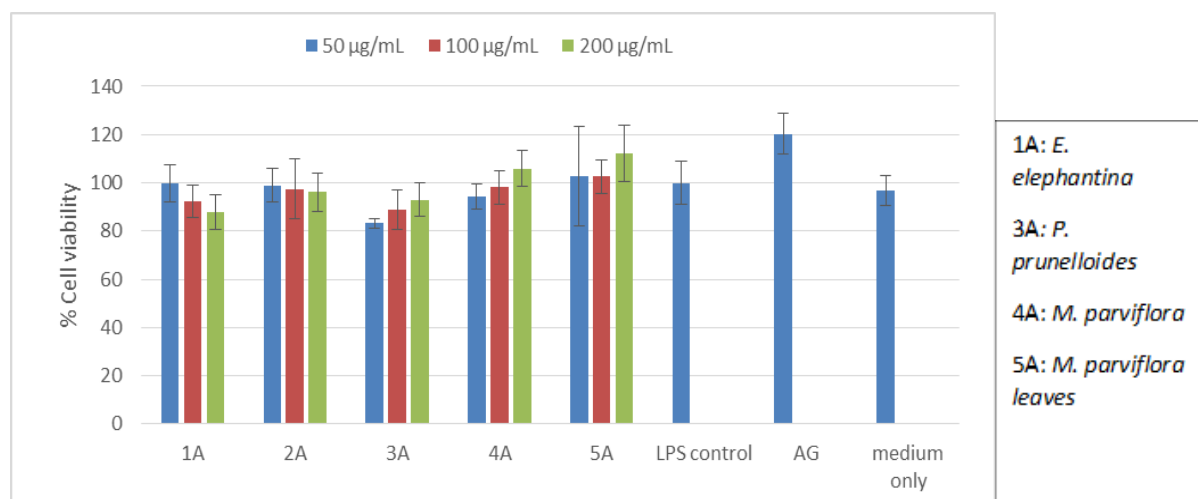
Although inflammation is usually associated with a protective or healing response, many chronic diseases are characterised by persistent inflammation, which ultimately results in tissue dysfunction. For this reason, the anti- or pro-inflammatory activity of test samples must be considered within the context of wound healing. The mouse macrophage cell line, RAW 264.7, is a well-characterised and popular model to investigate the anti-inflammatory potential of test samples. Cells are cultured in multi-well plates and activated by exposure to LPS, which induces the expression of iNOS with concomitant NO formation. Changes in NO production are determined by measuring the levels of nitrate in the culture medium. Simultaneous evaluation of cell viability and reduced NO production is used to confirm the absence of cytotoxicity and the anti-inflammation properties of the plant extracts. Anti-inflammatory activity can also be assessed using lipoxygenase assay whereby the enzyme 5-lipoxygenase catalyses the oxidation of unsaturated fatty acids containing 1-4pentadiene structures. The enzyme has arachidonic acid as its substrate, but it can also accept linoleic acid. Linoleic acid is converted to hydroperoxy linoleic acid, which is then evaluated by the appearance of a conjugate diene at 234 nm using a spectrophotometer. The 5-lipoxygenase activity is then evaluated by the by-product at 234 nm. A decrease in the initial reaction rate is an indication of the inhibitory activity of the sample (Baylac and Racine, 2003).



Bar graphs represent triplicate values of one experiment. Error bars represent the SD of the mean.

Figure 4.4: NO production in lipopolysaccharide (LPS)-activated macrophages treated with different concentrations (50, 100, and 200 $\mu\text{g/mL}$) of *E. elephantina*, *P. prunelloides*, *M. parviflora*, and *M. parviflora* leaf extracts. Statistical analysis was

determined to be * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.01$; ° $p < 0.05$, °° $p < 0.005$, °°° $p < 0.001$, °°°° $p < 0.01$; • $p < 0.05$, •• $p < 0.005$, ••• $p < 0.001$, •••• $p < 0.01$ compared to LPS.



Bar graphs represent triplicate values of one experiment. Error bars represent the SD of the mean.

Figure 4.5: Cell viability (%) of LPS-activated macrophages after 24 hours of exposure to treatments

The viability of RAW 264.7 cells treated with various concentrations (50, 100, and 200 µg/mL) of *E. elephantina*, *P. prunelloides*, *M. parviflora*, and *M. parviflora* leaf extracts for 24 hours was tested by the MTT assay. No extract demonstrated cytotoxicity against the RAW 264.7 macrophages in the range of 50, 100, and 200 µg/mL (see Figure 4.5). The RAW 264.7 cells were then treated with 50 µg/mL LPS to induce NO production. After 24 hours, nitrite concentrations were determined as an indicator of the NO production. As shown in Figure 4.5, *E. elephantina* significantly inhibited the LPS-induced production of NO, although not to the extent of the positive control aminoguanidine, which has been used in previous study by Saadat et al 2019, to show anti-inflammatory properties. *E. elephantina* therefore showed the most promising anti-inflammatory activity of all the tested plant extracts. Since *E. elephantina* inhibited the production of NO, which causes vasodilation and cellular migration, and because it reduces inflammation, it may be a good therapeutic target to promote wound healing.

4.7 TUBE FORMATION ASSAY

The tube formation is a crucial component of angiogenesis, and it plays a vital role in the wound-healing process by facilitating the development of new blood vessels, ensuring proper blood supply, and supporting tissue repair and regeneration. During wound healing, the body initiates a complex series of events to repair the damaged tissue. One crucial aspect of this process is the formation of new blood vessels and the active migration of cells in the region closest to the wound edge, which promote wound closure.

Cell tube formation is a critical step in angiogenesis, where endothelial cells, which line the inner surface of blood vessels, organise and align to create tubular structures. These tubes eventually mature into functional blood vessels that deliver blood to the wound site, which promotes tissue regeneration and repair.

The process of angiogenesis is essential for healthy tissue growth and wound healing. The dynamic interplay between wound healing and angiogenesis is essential for successful tissue repair. The formation of new blood vessels from pre-existing vessels is essential for many processes, including wound healing, in which newly formed blood vessels lined by endothelial cells supply O₂ and nutrients to the tissue, promote immune surveillance by haematopoietic cells, and remove waste products (DeCicco-Skinner et al., 2014; Beyer et al., 2018). As the wound matures, the angiogenic process is tightly regulated, which leads to a balance between vessel growth and regression. Once the wound is adequately healed, angiogenesis subsides, and the blood vessels assume a stable state to support the normal functioning of the repaired tissue. Disruptions in the delicate balance of angiogenesis can lead to impaired wound healing, such as chronic wounds, where angiogenesis is insufficient to support the repair process.

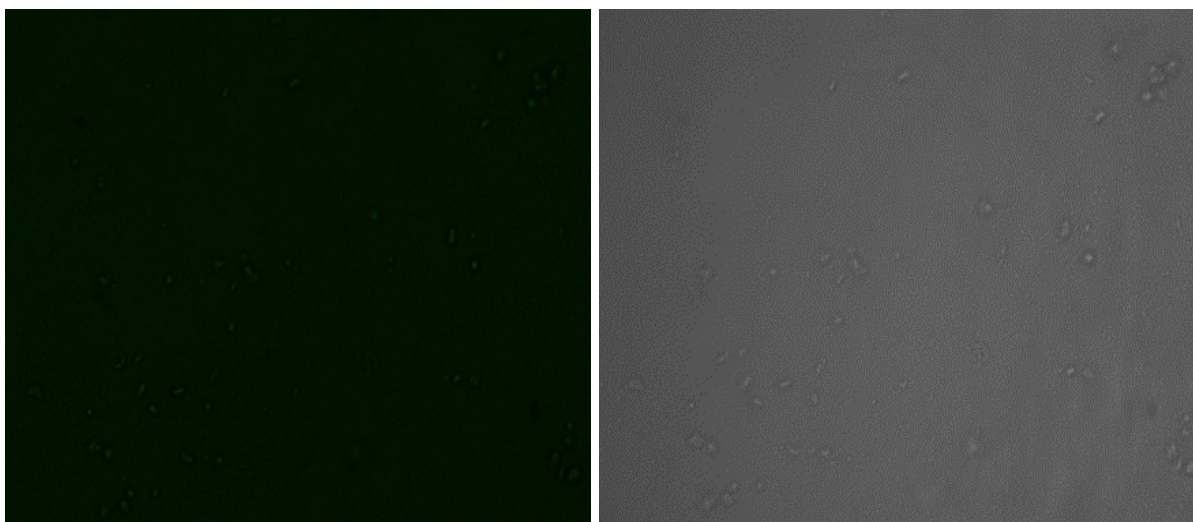


Figure 4.6: HeLa endothelial cell tube formation following treatment with *E. elephantina* aqueous extract on day one

Studies have reported that medicinal plants and their bioactive ingredients exert pro-angiogenic activity that promotes wound healing (Li et al., 2022) and, as such, *E. elephantina* was evaluated for wound-healing potential using the tubular formation assay. *E. elephantina* was the only selected plant extract for this assay since it demonstrated good antioxidant and anti-inflammatory properties and was less cytotoxic than the other plant extracts tested in this study. The results obtained, as shown in Figure 4.7, demonstrate the angiogenic factors secreted by the *E. elephantina* extract that are capable of inducing tube formation over time after 72 hours of treatment, as compared to Figure 4.6, where the cells were scattered and there was no tube formation yet. Endothelial cells migrated, grew, and began to form small branches within the 72 hours of plating.

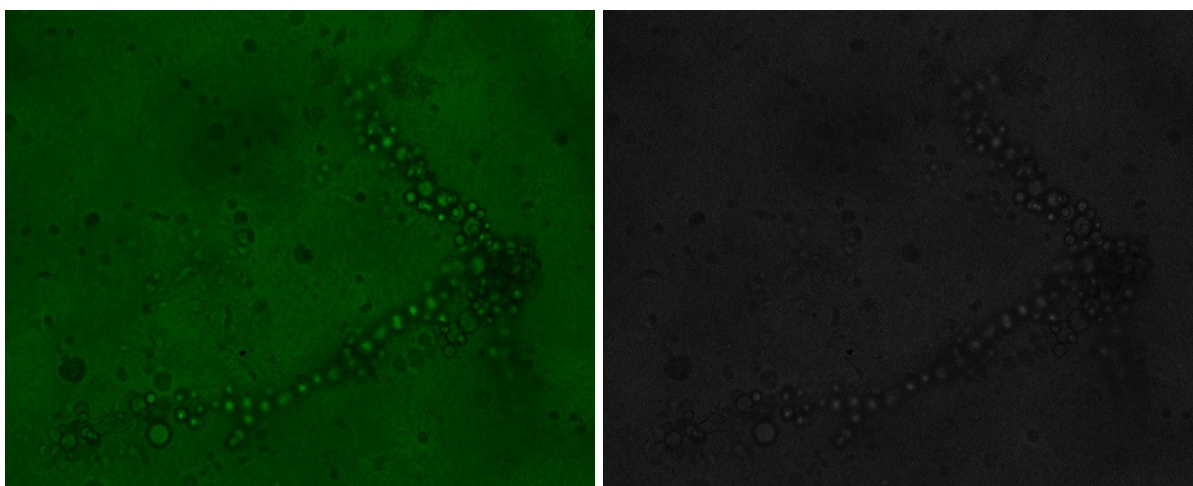


Figure 4.7: HeLa endothelial cell tube formation following treatment with *E. elephantina* aqueous extract on day three.

The ability of the *E. elephantina* extract to induce tube formation cells can be attributed to its phytochemicals, such as flavonoids, which have been reported to regulate the expression of VEGF, MMPs, and epidermal growth factor receptors (EGFRs). These angiogenic factors then initiate the formation of granulation tissue and angiogenesis is able to contribute to wound healing (Sahib et al., 2010). A study by Hong et al. (2009) determined the angiogenic activity of total saponins from the medicinal plant *Panax notoginseng* as an in vitro model for studies on angiogenesis. Their study showed that the extract stimulates proliferation and tube formation by the cells. These findings corroborate this study's findings on the *E. elephantina* extract (see Figure 4.7), where it was observed that the extract was able to stimulate migration and proliferation of the endothelial cells to form a vascular tube formation, which is an important process in wound healing.

The saponins present in the *E. elephantina* extract could also be responsible for the stimulation of VEGF, which is considered to be the predominant and the most effective angiogenic mediator in human cutaneous wounds (Roy et al., 2008). Anthraquinones that are present in the *E. elephantina* extract were identified as angiogenic factors that may be beneficial to the wound-healing process (Majewska & Gendaszewska-Darmach, 2011). Anthraquinones are responsible for the formation of new blood vessels and the expression of proteins related to angiogenesis, vWF, VEGF, VEGF receptor Flk-1, and blood vessel matrix laminin (Chang et al., 2002; Majewska & Gendaszewska-Darmach, 2011). In this study, it is evident that the *E. elephantina* aqueous extract contributes to angiogenesis as it induced tubular formation of HeLa cells. *E. elephantina* therefore has beneficial effects for the promotion of wound healing; however, further studies to elucidate the mechanism of action and the identification and isolation of the active ingredient(s) from this plant are still needed.

CHAPTER 5:

CONCLUSIONS, LIMITATIONS, AND RECOMMENDATIONS

5.1 CONCLUSIONS

The purpose of this study was to evaluate extracts prepared from *M. parviflora*, *E. elephantina*, *D. anomala*, and *P. prunelloides*, which have been reported in the literature and anecdotally described as playing a role in the treatment of microbial infection, inflammation, and oxidation, all of which are essential for effective wound healing. Saponins, alkaloids, tannins, anthraquinones, terpenoids, and steroids were screened from methanol and aqueous extracts of *P. prunelloides*, *E. elephantina*, *D. anomala*, and *M. parviflora*. Saponins, flavonoids, tannins, glycosides, and steroids were identified in all the plant extracts; however, the alkaloids, terpenoids, and anthraquinones identified varied according to their respective plant extract, as seen in Table 4.1. Saponins, phenolic compounds, and tannins are known for their anti-inflammatory properties (Molefe, 2013), while terpenoids are known for their antibacterial properties (Mahmood et al., 1993). The presence of these phytochemicals in *E. elephantina*, *P. prunelloides*, and *M. parviflora* contributed to their observed antimicrobial and anti-inflammatory activities as reported in this study. In general, phytochemicals that possess many beneficial properties, such as those with antibacterial, antifungal, antioxidant, and anti-inflammatory activities, have the potential to be successful in at least in one stage of the wound-healing process (Thakur et al., 2011).

A generally very low cytotoxic effect was observed for all the methanol and aqueous extracts at both low and high concentrations (50 and 200 µg/mL) for all the plant extracts. *D. anomala*, however, showed cytotoxicity even at low concentrations for both the methanol and aqueous extracts; it was therefore excluded from any further tests. The antimicrobial experiment revealed that most of the tested extracts could inhibit the tested microorganisms, although some at higher concentrations of 200 µg/mL. The aqueous extract prepared from *M. parviflora* had the greatest efficacy against *P. aeruginosa* at an MIC of 50 µg/mL, with an inhibition zone of 28 mm. The extracts may therefore be effective for treating infected wounds to prevent chronic wounds and to promote the wound-healing process.

The *M. parviflora* whole plant extract and the *M. parviflora* leaf extract exhibited the same antioxidant properties as catechin, as shown in Figure 4.2. The antioxidant properties of the *M. parviflora* extract indicated that the plant offers wound protection against oxidative damage and thus promotes wound healing. The results obtained for *E. elephantina* from the tube formation assay revealed novel information about the plant extract and its wound-healing ability. The results in Figure 4.7 showed that the angiogenic factors secreted by the *E. elephantina* extract are capable of inducing tube formation over time, as well as the migration of endothelial cells, which are essential for wound healing.

This study observed that *E. elephantina*, *M. parviflora*, and *P. prunelloides* demonstrated good antioxidant and anti-inflammatory properties and therefore are promising candidates for new wound-healing agents. Positive correlations were shown between anti-inflammatory activity and wound closure by Pringle et al. (2021), who indicated a relationship between inflammation and wound healing, which means that one factor affects another or relies on the other. It was observed that all the tested plants contained the chemical components that were examined and mentioned above, which are known to play a major role in the plants' antioxidant and anti-inflammatory effects and have additionally been documented to contribute to the wound-healing potential of the plants *E. elephantina*, *M. parviflora*, and *P. prunelloides*.

5.2 LIMITATIONS AND RECOMMENDATIONS

The study was able to identify several phytochemical compounds in the extracts; however, the existence of these compounds could not be validated using additional methods such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) as it was not in the scope of this study to perform TLC and HPLC. Subsequent experiments are therefore recommended to use these methods to validate the existence of these active compounds in the extracts. In addition, the compounds' activities were only assumed to be examined since they were not subjected to any direct testing in this investigation. Future research should therefore focus on the bio-assay-guided isolation of the detected compounds, as well as any additional new compounds that may be present. In order to verify the function that these compounds play in the activity of the plant extract, they should also be subjected to direct testing. Experiments designed to test for antimicrobial properties were

conducted with the aim of identifying extracts that exhibited moderate to high activity against various microorganisms associated with wound infections.

Future experiments should include the bio-autographic assay, which will evaluate the antimicrobial properties of isolated compounds. This will make it possible to determine all the active extracts, the purification of which may lead to the identification of further active chemicals. In addition, this research only examined the effects of the extracts on four different microorganisms. This needs to be broadened to include a wider range of species, including other species of yeasts instead of only one (*C. albicans*). In the study, extracts were evaluated for cytotoxicity for a maximum of 48 hours. Although this allowed the extracts to exhibit short-term cytotoxicity, their long-term effects on cells could only be assumed. To determine the extent of cytotoxicity, extracts must be evaluated for a longer duration of up to 72 hours. Intracellular ROS was measured using CellROX as a fluorescent marker.

The results for tube formation assay were obtained with a Leica microsystems CMS GmbH® microscope at x 20 magnification. The microscope lens did not have eye-piece graticule to measure the size of the tube as it was forming. Due to limited resources, it was not possible to get access to a microscope that has an eye-piece graticule, therefore the results were solely relied on visual observations to draw conclusions. To quantify the tube network in future studies several different ways can be used to measure the number of tubes; number of loops/meshes; number of branch sites/nodes; length of tubes. Additionally, computer program such as Image J with the Angiogenesis Analyzer plugin¹⁶ for quantification of tube networks can be utilized. . Lastly, only one inflammatory assay, NO inhibitory activity, was conducted in this study. Since chemical entities could exhibit anti-inflammatory activity through various mechanisms, extracts should be explored for their ability to inhibit other inflammatory processes such as cyclooxygenase and cytokine release. To investigate the effects of the extracts on cellular proliferation and migration, the fibroblast proliferation assay and scratch wound assay were required as part of this study. Unfortunately, the scratch wound assay could not be conducted as fibroblast cells could not be optimised even after numerous attempts. The tubular formation assay was thus performed to determine the ability of *E. elephantina* to promote angiogenesis through the migration of cells leading to tubular formation since there is a link between angiogenesis and

wound healing. Additionally, further research should be conducted using wounds induced in laboratory animals, as they are more physiologically related to humans.

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