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**THE USE OF TRADITIONAL MEDICINAL  
PLANTS FOR TREATING DERMATOLOGICAL  
DISEASES AND WOUND HEALING IN  
KWAZULU-NATAL, SOUTH AFRICA**

**By**

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Submitted in fulfilment of the academic requirements for the Degree of  
Doctor of Philosophy

**Research Centre for Plant Growth and Development  
School of Life Sciences  
University of KwaZulu-Natal, Pietermaritzburg  
South Africa**

**April 2018**

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# STUDENT DECLARATION

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## THE USE OF TRADITIONAL MEDICINAL PLANTS FOR TREATING DERMATOLOGICAL DISEASES AND WOUND HEALING IN KWAZULU-NATAL, SOUTH AFRICA

I, **Shanaz Ghuman**, student number: **953021782** declare that:

- (i) The research reported in this dissertation, except where otherwise indicated, is the result of my own endeavours in the Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg;
- (ii) This thesis has not been submitted for any degrees or examination at any other university;
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SIGNATURE

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SHANAZ GHUMAN

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We hereby declare that we acted as Supervisors for this PhD student:

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the College of Agriculture, Engineering and Science, Higher Degrees Office for examination by the University appointed examiners.

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## DECLARATION 2 – PUBLICATIONS

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**Shanaz Ghuman<sup>a</sup>, Bhekumthetho Ncube, Jeffrey F. Finnie, Lyndy J. McGaw, Roger M. Coopoosamy, Johannes Van Staden.** Antimicrobial activity, phenolic content and cytotoxicity of medicinal plant extracts used for treating dermatological diseases and wound healing in KwaZulu-Natal, South Africa - *Frontiers in Pharmacology* 7, 1-19. September 2016, Article 320.

Contributions: Review of literature, experimental work and manuscript preparation were performed by the first author under supervision by the rest of the authors.

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# CONFERENCE CONTRIBUTIONS

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## **Research Centre for Plant Growth and Development Annual Meeting Presentations**

2012 - *In vitro* studies of wound healing activities of medicinally important plants found in KwaZulu-Natal, South Africa.

2013 - Antimicrobial and phytochemical screening of selected species of the medicinally important genus *Aloe*, KwaZulu-Natal, South Africa.

2014 - Antibacterial screening of medicinal plants for healing skin ailments in KwaZulu-Natal province, South Africa.

2015 - Antimicrobial screening, phenolic content and cytotoxicity of medicinally important plant extracts commonly used for treating skin, dermatological diseases and wound healing in Kwazulu-Natal, South Africa.

2016 - Anti-inflammatory, antioxidant and wound healing properties of South African medicinal plants.

2017 - Anti-inflammatory and wound healing properties of South African medicinal plants.

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# ABSTRACT

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Medicinal plants used for wound healing and skin diseases are the key to unlocking the doors to combating resistance of pathogens to pharmaceuticals and allopathic management. This study was aimed at investigating the antimicrobial efficacies, phenolic content, cytotoxicity and antioxidant effects, as well as the anti-inflammatory and wound healing capacity of traditional medicinal plant extracts commonly used for treating skin conditions and wound healing in traditional medicine within KwaZulu-Natal. A preliminary survey was conducted at the Umlazi and Durban herbal markets with traditional healers and eleven medicinal plants commonly used for dermatological conditions were identified and selected. These medicinal plants were obtained with the assistance of an experienced horticulturalist at the Silverglen Nursery Conservation Garden, Durban, KwaZulu-Natal. The antimicrobial activity of the selected plant extracts were tested against common disease-causing skin microorganisms; seven Gram-positive bacterial species (*Actinomyces brasiliensis*, *Micrococcus* species, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus epidermidis* and *Bacillus subtilis*); seven Gram-negative bacterial species (*Proteus vulgaris*, *Proteus mirabilis*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes*) and four fungal species (*Candida albicans*, *Candida tropicalis*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*). The pathogenic strains used in the study are the ones associated with wound healing problems. It has been reported that there are more than 250 000 species of plants, however, only 5-10% have been studied chemically for the presence of biologically active compounds. Natural-derived compounds are reported to be the most relevant compounds for future drug discovery, yet the biological activity of much of the world's biodiversity remains scientifically untested. This is particularly true for many indigenous traditional medicines of South Africa. In rural communities of South Africa, the treatment of various skin conditions such as burns, bruises, cuts and scratches, when left untreated at the initial stages, result in consequences such as microbial infections, sepsis and in more severe cases death.

Eleven plant species were selected and separated into different plant parts (bulbs, roots and leaves) and extracted with different solvents (methanol, chloroform, dichloromethane, acetone and methanol). The extracts were assessed for their antimicrobial activity using disc diffusion and microdilution techniques. Extracts from *Aloe ferox*, *A. arborescens* and *Hypericum*

*aethiopicum* were the most active against almost all of the tested bacterial and fungal strains. All plant species exhibited some degree of antimicrobial activity. The observed antimicrobial efficacy demonstrated by some plant species and the general lack of cytotoxic effects of all the tested extracts presents some promising and beneficial aspects of these medicinal plant extracts in the treatment of skin and wound infections. The *Aloe* species, *A. ferox*, *A. arborescens*, *A. aristata* and *H. aethiopicum* were among the best extracts that exhibited consistently good antimicrobial activity and warrants further investigations and possible isolation of bioactive principles.

Total phenolic levels, flavonoids and tannins were also higher for *A. ferox*, followed by *A. arborescens*, *A. aristata* and *H. aethiopicum* respectively. The cytotoxicity results of all plant extracts were in the range of 90-100% survival after 24 h in the Brine shrimp assay. Extracts considered lethal had > 50% shrimp death. The MTT cytotoxicity test yielded LC<sub>50</sub> values of > 1 mg/mL for all extracts indicating that the extracts were not cytotoxic. The extracts were assessed for anti-inflammatory activity using the nitric oxide release and lipoxygenase inhibition assays. All plant species exhibited some degree of anti-inflammatory activity. The observed antioxidant results (DPPH, FRAPS, CLAMS) was significant for many of the plants. In the anti-inflammatory assays the LOX revealed that five of the medicinal plant extracts, *Bulbine natalensis*, *Eucomis autumnalis*, *Hypericum aethiopicum*, *Tetradenia riparia* and *Zantedeschia aethiopica* were effective as anti-inflammatory agents with IC<sub>50</sub> values below the quercetin control and ranged from 3.55±0.11 to 9.52±0.11 µg/ml.

Plant extracts are associated with several antimicrobial properties including anti-inflammatory and antioxidant. The use of bioactive extracts or phytochemicals from a variety of plants contributes to public health care. The interplay between free radicals and antioxidants is important in maintaining health, ageing and age-related diseases. Free radicals generated in excess may accumulate and outweigh the protective effects of antioxidants. Values of the IC<sub>50</sub> for the flavonoid control, quercetin was 6.30 µg/ml. Plant extract values were considered in relation to this control. Those values greater than 100 µg/ml indicated less active anti-inflammatory agents and plant extracts that were within this range in the study were the leaf extracts of the three *Aloe* species (*A. arborescens*, *A. aristata*, and *A. ferox*) and *H. limifolia*. There seem to be a marked difference between the leaf and the stem extracts of *T. riparia* in terms of the inhibition potential of NO with the stem demonstrating an almost seven-fold difference to that of the leaf extract at an IC<sub>50</sub> value of 13.99 µg/ml. Further significant IC<sub>50</sub> values were observed for *H. aethiopicum* leaf extract (22.47 µg/ml) and *M. plumbea* extract for

the leaf at 29.35  $\mu\text{g/ml}$ , the bulb at 22.04  $\mu\text{g/ml}$  and the root extract at 26.93  $\mu\text{g/ml}$ ). In addition, the *Bulbine* species were observed to have an  $\text{IC}_{50}$  value for *B. natalensis* root extract at 28.64  $\mu\text{g/ml}$  and *B. frutescens* leaf extract at 26.32  $\mu\text{g/ml}$  showing significant anti-inflammatory characteristics towards NO inhibition.

The results of the wound healing assay/protein precipitating activity were significant for the three *Aloe* species, two *Bulbine* species and support scientific evidence from previous research. In addition, medicinal plant extracts from *E. autumnalis*, *H. limifolia*, *H. aethiopicum*, *T. riparia*, and *Z. aethiopica* demonstrated promising and beneficial results for potential use in the treatment of skin diseases and wound healing. Antioxidant assays as well as anti-inflammatory assays (nitric oxide release and lipoxygenase inhibition assays) and wound healing assays supports the dermatological and wound healing usage of these traditional medicinal plants and warrants further investigations and possible isolation of bioactive principles. The protein-precipitating capacity as a wound healing model was significant for *Haworthia limifolia*  $82.71 \pm 0.74$  % while the rest of the plant extracts had moderate to low values.

Overall, the results from this multi-dimensional medicinal plant study provide extensive information on the eleven plant species and their various plant parts. Science needs to strengthen the multidisciplinary research mechanisms to support the evaluation of medicinal plants and effectively facilitate collaboration between traditional and conventional health practitioners.

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# LIST OF ABBREVIATIONS

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<b>ANT</b>	Antioxidant activity
<b>ATCC</b>	American Type Culture Collection
<b>AU</b>	African Union
<b>BHA</b>	Butylated hydroxyanisole
<b>BHT</b>	Butylated hydroxytolulene
<b>CFU</b>	Colony forming units
<b>CLAMS</b>	$\beta$ - carotene linoleic acid bleaching assay
<b>COX</b>	Cyclooxygenase
<b>CTE</b>	Catechin equivalents
<b>DCM</b>	Dichloromethane
<b>DMEM</b>	Dulbecco's Modified Eagle's Medium
<b>DMSO</b>	Dimethyl sulfoxide
<b>DNA</b>	Deoxyribonucleic acid
<b>DPPH</b>	2, 2-Diphenyl-1-picrylhydrazyl
<b>DW</b>	Dry weight
<b>EC</b>	Enzyme Commission
<b>FCS</b>	Foetal calf serum
<b>FDA</b>	Food and Drug Administration
<b>FRAP</b>	Ferric reducing antioxidant power
<b>GAE</b>	Gallic acid equivalents
<b>HCl</b>	Hydrochloric acid
<b>INT</b>	p-iodonitrotetrazolium
<b>KCl</b>	Potassium chloride
<b>KZN</b>	KwaZulu-Natal
<b>LCE</b>	Leucocyanidin equivalents
<b>LOX</b>	Lipoxygenase
<b>LPS</b>	Lipolysaccharide
<b>MEM</b>	Minimal essential medium
<b>MH</b>	Mueller Hinton
<b>MIC</b>	Minimum inhibitory concentration
<b>NaCl</b>	Sodium chloride

<b>NaNO<sub>2</sub></b>	Sodium nitrate
<b>NO</b>	Nitric oxide
<b>NOS</b>	Nitrous Oxide Systems
<b>PBS</b>	Phosphate buffer solution
<b>PSF</b>	Penicillin/streptomycin/fungizone
<b>ROS</b>	Reactive oxygen species
<b>RSA</b>	Radical scavenging activity
<b>SA</b>	South Africa
<b>SDS</b>	Sodium dodecyl sulphate
<b>SG</b>	Shanaz Ghuman
<b>TBHQ</b>	Tertbutyl-hydroquinone
<b>UK</b>	United Kingdom
<b>UKZN</b>	University of KwaZulu-Natal
<b>USA</b>	United States of America
<b>UTI</b>	Urinary tract infections
<b>UV</b>	Ultraviolet
<b>WHO</b>	World Health Organization

# CHAPTER ONE

## INTRODUCTION AND LITERATURE REVIEW

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### 1.1 Introduction

Indigenous plants are a source of traditional medicines since the existence of mankind to treat, manage and prevent various health conditions (**DOLD and COCKS, 2002a; 2002b**). Traditional medicinal plants form part of the principal health care management systems for a significantly large number of the lower income populations in most developing countries. There is a worldwide demand for the many bioactive compounds produced by medicinal plants. A significant proportion of South Africa's population is known to have a high preference for traditional medicinal health care systems over and above the allopathic or conventional health care management systems (**MANDER, 1997; 1998; DOLD and COCKS, 2002a; 2002b; MOENG and POTGIETER, 2011; WILLIAMS et al., 2013**). A broad spectrum of indigenous therapeutic plants are used for herbal medicines as they are abundant in natural bioactive products and metabolites such as the flavonoids, tannins, alkaloids and terpenoids (**MANDER, 1997; 1998; DOLD and COCKS, 2002a; 2002b; MOENG and POTGIETER, 2011; WILLIAMS et al., 2013**). The practical assessment of the contents of medicinal plants for biological activity is important to validate their use and relevance particularly by traditional healers.

Medicinal plants are utilised for the management and prevention of an extensive range of disease conditions. Many rural populations are prone to sores, wounds and other skin-related conditions and are without access to conventional health care thus relying mainly on traditional health care remedies (**DOLD and COCKS, 2002a; 2002b**). A notable factor in rural communities is that many of the treatments are sought and sourced from fresh or partially dried plant material (**MOENG and POTGIETER, 2011; WILLIAMS et al., 2013**). In urban communities relying on traditional medicines, the traditional herbal market is the source of these plant materials. Medicinal plant material may be stored in these markets over long periods. The effectiveness of these dried materials needs to be regularly checked/monitored for efficacy and potential toxicity as shelf life diminishes with time (**MANDER, 1997; 1998; DOLD and COCKS, 2002a; 2002b; MOENG and POTGIETER, 2011; WILLIAMS et al., 2013**).

Wounds are known as any form of damages to the body that may be the consequence of an opening or breakage in the skin. Healing ensues in a complex but well organized sequence of events involving the interaction of many cytokines and cell types (**LI and LI, 2003**). Chronic wounds are of major concern affecting many patients, significantly and gradually reducing their value of life (**LI and LI, 2003**). It has been recorded that an estimated six million people are sufferers of chronic wounds worldwide (**KUMAR et al., 2007a; 2007b; COETZEE et al., 2010**). Chronic wounds by definition, are any damages that occur in the layers of the mammalian skin coherence when this breakage persists for longer than six weeks or is due to infection and non-healing recurs frequently. The leading reasons for these non-healing wounds include diabetic complications of the skin, post-operative complications from surgery, vascular insufficiency due to blockages and obstructions and skin damage due to pressure. According to **KUMAR et al. (2007a)** and **COETZEE et al. (2010)**, chronic wounds are known to affect four million persons in Germany and almost three million patients between the ages of 45 and 65 years in the United States of America (USA). The prevalence is 120 per 100,000 of the total population in these countries, increasing to a further 800 per 100,000 in patients of ages 75 years and older of the total population (**KUMAR et al., 2007a; COETZEE et al., 2010**). The cost to healthcare systems in the management of consequences of untreated wounds and sores is enormous, amounting to billions of Dollars. Almost two percent of the yearly health maintenance budgets of Germany, France and the United Kingdom are spent on the treatment of venous leg ulcers and thrombosis (**JÄRBRINK et al., 2016**). The USA spent almost two and half billion Dollars in 2005 on advanced wound care products and in Germany, the budget was five billion Euros yearly (**JÄRBRINK et al., 2016**). There are major impacts on the economy of many countries as wound lesions are socially isolating, taking an excessive length of time to heal and the rate of recurrence is more regular. The reduced value of life experienced is notably experienced by these patients and their relations. Even though the frequency of enduring wounds and wound care training is high, the management thereof is regarded as inadequate in Germany and the USA (**KUMAR et al., 2007a; COETZEE et al., 2010**). A study in Canada reported that many doctors, specifically family physicians, perceive themselves as unprepared in the management of conditions like pressure ulcers, suggestive of the fact that they do not receive an adequate amount of training for the consequences of this wound disorder. The Canadian nurses also indicated low levels of confidence in the familiarity of medical doctors who administered and would oversee the treatment of chronic wounds in patients who are presented at the health care facilities (**JÄRBRINK et al., 2016**).

Nursing personnel in South Africa, especially in primary health care services where ethics of care differ, are left responsible for patient chronic wound care and are known not to, nor frequently, seek help from the medical doctors (**JÄRBRINK et al., 2016**). The incorrect application of wound care and management frequently can lead to lengthy hospital admittances and extended therapeutic duration leading to depletion of constrained resources. Wound management training promotions have favourable effects on the use of assets and consequences for patients such as length of hospital stay and period to reach wound healing (**JÄRBRINK et al., 2016**). Chronic wounds execute a significant and unappreciated encumbrance on society as a whole, the national healthcare system and the individual. The knowledge of incidence and the prevalence of chronic wounds in conjunction with population characteristics is significant for advising resource allocation and healthcare planning. According to **LALL and KISHORE (2014)**, the field of modern biomedical sciences research, is emerging the fastest in the area of wound healing management. In South Africa, many traditional specialists have valuable knowledge of less known wild plants used by healers for treating burns and wounds. Different pharmacological models and scientific tests on animals using various medicinal plants have been conducted with screening and evaluation of their wound healing properties (**LALL and KISHORE, 2014**). However, the capacity for using many of these medicinal plants remains largely unknown. Wound healing is important for the rebuilding of damaged tissues and correction of the malfunctioned status of the skin when damaged. This is a composite and complicated process commencing in response to any damage and proceeds to restore functionality and integrity of injured tissues (**BEGUM and NATH, 2000; GOVINDARAJAN et al., 2007**). Initial wound healing begins with agglutination which results in clotting of blood and scab formation to prevent the loss of blood. The ability to clot blood is of importance as this would indicate the ability of plant extracts to aid in wound healing. Collective processes for healing requires the interaction of various tissues and tissue lines. The process is an inflammatory response to injury and involves blood clotting, platelet aggregation and the formation of fibrin. An alteration occurs in the matrix substances and constituents, re-epithelialisation and angiogenesis. Completed healing will only be achieved once the damaged frontages are sealed by a collagen network (**BEGUM and NATH, 2000; GOVINDARAJAN et al., 2007**).

The intricate value of supreme wound healing is to decrease damage to the tissues and increase acceptable tissue oxygenation and blood flow levels, proper nutrition and a moist wound healing surrounding in order to re-establish the anatomical competence and functioning of those areas and parts affected (**KARODI et al., 2009**). According to **MACKAY and MILLER**

(2003), the key objective of wound management is to heal the injury in minimal time with least pain and discomfort. The requirement at the site of the wound is that the wound ought to have an elastic acceptable scar with the best ductile strength while the critical aim is to speed up wound healing. Scientists continue to use medicinal plant extracts to endorse the various phases of wound healing which are both successive and concomitant. These may include inflammation, coagulation, collagen formation, fibroblast proliferation, re-epithelialization, cellular growth and wound contraction. Medicinal plant extracts that promote fibroblast proliferation and collagen formation commonly used for wound healing in South Africa include *Calotropis procera* (RASIK et al., 1999), *Aloe vera* (CHITHRA et al., 1998a; 1998b; LEE and MOON, 2003; SUBRAMANIAN et al., 2006; 2011) and *Centella asiatica* (SHUKLA et al., 1999).

According to a study by PURNA and BABU (2000), traditional plant medicines used in wound management do assist in the elimination of the foreign objects or damaged tissue from the wound or debridement. These also provide humidity to increase the formation of an appropriate environment for natural disinfection and the healing process. Most infections are a key reason for the high mortality and morbidity rates in wounded patients. Plant extracts may prevent or reduce infections that leads to high risk of sepsis and in so doing can reduce perpetuation of the inflammatory phase. Future research on the fractionation of bioactive compounds and the synergistic effects of plant extract components on pathogenic microorganisms will be able to make available an improved understanding of skin infection management in the process of wound healing (KARODI et al., 2009). A study by MUKHERJEE et al. (2003) on two herbal formulations evaluated for wound activity using both the excision and incision methods found that significant responses in both models occurred. SUBRAMANIAN et al. (2006; 2011) reported the action of *Aloe vera* leaf gel in its capacity for sealing properties and wound healing.

Plants are known to contain and yield diverse chemical substances that are active in the human body. Scientific developments have isolated and evaluated numerous medicinal properties for a large number of plants including the extraction, isolation and analysis of their healing constituents. Traditional plant medicines are extensively used by African people and some plants used are known to have pharmacologically active compounds. Medicinal plants have become the attention of many studies due to the critical need for the evaluation of traditional methods of treatment with significant importance placed on the screening for active products (SPARG et al., 2002). In South Africa; a country where an opulent plant diversity exists, there is a strong demand for medicinal plants and it has been found that the traditional usage in health

care is high (JÄGER et al., 1996; MANDER, 1997; 1998). About 70% of the indigenous population in South Africa consult traditional healers for primary health-related sicknesses, preferring accepted herbal medications, many of which come from indigenous plant by-products (JÄGER et al., 1996; MANDER, 1997; 1998). Medicinal plants may be acquired from either the herbal markets, gathered in the wild and/or from traditional healers. The challenge is that several of these medicinal plants are endangered or threatened species and thus indigenous species populations are decreasing due to extreme gathering for medicinal use which is characteristic for emerging countries (FARNSWORTH, 1984; SRIVASTAVA et al., 1996). Both developed and traditional applications are inclined towards the advancement in the recording of safe medicinal products. These processes are essential in the management of a broad range of disorders especially those due to various disease causing microorganisms becoming resilient to clinical treatment (AHN et al., 2001).

Numerous plant species have been documented and acknowledged in the treatment of cracked skin, rashes, burns, insect bites, boils, mouth blisters and cold sores (RAINA et al., 2008; VAN WYK et al., 2009; NAGORI and SOLANKI, 2011). A number of screening and extraction procedures to isolate naturally active medicinal compounds have been established for distinguished medicinal plant species, many have revealed antimicrobial capacity (RABE and VAN STADEN, 1997; GRIERSON and AFOLAYAN, 1999a; 1999b; McGAW et al., 2000; AFOLAYAN et al., 2002; MATHABE et al., 2006; COOPOOSAMY and MAGWA, 2007; LATEGAN et al., 2009; DE WET et al., 2010). Systematic authentication for the separation of antifungal and antimicrobial products from plants has been highlighted for additional research whilst others are registered and patented productively (FARNSWORTH, 1994; FOX, 1999; OFODILE et al., 2010). Traditionally, plants are a rich source of antimicrobial activity against various infectious agents. An isoquinoline alkaloid called emetine found in *Cephaelis ipecuanha* and related species have been used for a long time as an amoebicidal drug in the treatment of inflammation caused by *Escherichia histolytica* infections (ABDULLAH et al., 2009; KHORSHID et al., 2010; PATHER et al., 2011). An alkaloid called quinine, naturally occurring in the bark of *Cinchona* trees, is used to treat malaria. Phytochemicals studied throughout the world are known for their inhibitory effects on several types of microorganisms both *in vivo* (ABDULLAH et al., 2009; KHORSHID et al., 2010; PATHER et al., 2011) and *in vitro* wound healing (COWAN, 1999).

Bioactive compounds of medicinal plants identified for use as therapeutic agents were isolated for further investigation and have been used in the development of therapeutic drugs. The



compounds of known structures were used to produce patentable entities of higher medicinal activity and lower toxicity (**FABRICANT and FARNSWORTH, 2001**). According to **BALANDRIN et al. (1993)**, **NAHRSTEDT (2002)** and **NAHRSTEDT and BUTTERWECK (2010)**, of 250 000 species of plants approximately 5-10% had only been studied chemically for the presence of naturally occurring active compounds. International research laboratories are involved with screening of plants with therapeutic potential for biological activity and yet the potential for novel medicines from plants is still underexplored (**HOSTETTMAN et al., 1996**). The evaluation of the plants selected for this study is necessary to validate their use by the traditional healers and also to possibly uncover potential leads for therapeutic drug development and/or herbal preparations.

## **1.2 Traditional Medicine**

The World Health Organisation (**WHO, 2000; 2002; 2008**) has published various reports on indigenous plant medicines which serve and highlight the health necessities of the world's inhabitants in the extensive pastoral regions of developing and non-industrialized countries. The WHO Indigenous Treatment Programme has defined traditional medicine as the totality of practices and knowledge that are applied in the analysis, eradication and prevention of social, mental and physical disparities (**RUKANGIRA, 2001**). Internationally people depend on medicinal plants for their day-to-day well-being and health management needs (**VAN WYK et al., 2009**). The main reasons for rural populations being involved in traditional health care amongst others include strong beliefs in tradition, low cost and easy availability of traditional medicines (**BANQUAR, 1993**). The insightful understanding of plant therapies in traditional beliefs has developed and evolved over countless years and verbally transmitted from one generation to the next generation of healers. Over time the understanding of indigenous therapies from generation to generation depends mostly on the accuracy, observations and experiences of the people. The differences and similarities between the traditional communities is why we have such diverse plurality in traditional medicine. Diverse plurality in traditional medicine are circumstances within society in which communities of different racial, ethnic, social or religious affiliations maintain a self-sufficient involvement in the development of their traditional cultural practices in health care (**HÖRBST et al., 2017**). Pluralism symbolizes a diversity of understandings or viewpoints rather than a single approach or method. This is the existence of multiple healing systems. Cultural diversity and plurality of healing systems can be seen as identical twins! (**HÖRBST et al., 2017**). Modern medical science and alternative traditions from Africa are examples of traditional knowledge of diverse plurality practices and

exist throughout the world in traditional medicinal systems of the Chinese, Buddhists, Indian and Islamic origin.

Historically, traditional systems throughout the world and in every region had its own unique combination of natural medicines. Tribes of Arabic, Chinese and African origin have their own traditional medicines rooted in their cultural contexts and vary from one to another (**VAN WYK et al., 2009**). In South Africa, Government has followed other developing countries by giving strong consideration to the importance of healthcare usage of indigenous medicines as an innovative course for the acknowledgement, venture and research strategy of programmes in Ethnopharmacology (**RUKANGIRA, 2001**). Plant-derived medicinal drugs are of economic and strategic significance for the African continent. Around 10% of the world's varied plant population is encompassed within the southern African continent and moderate to minor biochemical work has been done (**ELOFF, 1998a; 1998b**). Even though people reside in the world where numerous disease-causing microorganisms are managed, the advent of various medicinal resistant strains of disease causing organisms is motive for severe concern (**MADIGAN et al., 2015**). Currently, the preservation and conservation legislation in South Africa effective since April 2008, allows researchers to have unrestricted right to use natural resources as long as the researcher has a gatherer's permit and follows the terms and rules laid down. This allows the application and use of biological resources within regulation and control to avoid overexploitation (**VAN SCHALKWYK, 2009**). An integration of an export and bioprospecting permit is issued only when the minister responsible is satisfied that the processes are utilised for purposes that are in the interest of the public. These include economic development and conservation of South Africa's biodiversity, improving scientific understanding and technical capability of South Africa's people and relevant organizations (**VAN SCHALKWYK, 2009**). An extensive understanding of these plants is imperative as it has the probability to lead to findings of new alternate possibilities for the treatment of infections but also has the capacity of conservation of rare plant species that are overharvested and exported for traditional medicinal use.

An association or national council of traditional healers in South Africa was established in 1989, comprising minor organisations formed by the country's traditional healers with over 2000 registered members and is known as South African Healers Association (SOAHA). The NPO has steered various endeavours that resulted in the formation of regulatory and monitory authorities on traditional healers practices (**LECLERC-MADLALA, 2002; LOUW AND DUVENHAGE, 2016**).

They have practiced accomplishments through representation of experienced members and sustainability with collaborations and incorporation of various relevant stakeholders. The present regime intends to form associations between contemporary and indigenous medical systems. At present, in South Africa many organizations register and regulate the scope of practice of traditional healers (**LECLERC-MADLALA, 2002; LOUW AND DUVENHAGE, 2016**). If any plant types were discovered to be in danger and at risk due to an extensive requests for plants for medicinal use, these organizations can implement measures that ensure sustainability. Therefore it is imperative to record this information for prospective generations (**HUTCHINGS, 1989; HUTCHINGS and VAN STADEN, 1994; HUTCHINGS et al., 1996**). Amongst these communities, the indigenous women are known to have extensive understanding of medicinal plant usage due to their responsibilities as caregivers of youngsters at households (**KOTHARI, 2003**). In recognizing and restoring ethnic women's understanding through organizational provisions and care, their efforts in passing significant knowledge to future generations enhances the sustainability of the use of "natural capital" (**MIKKELSEN, 2005**). A study by **ZOBOLO and MKHABELA (2006)**, conducted in the rural parts of Eshowe, Northern KwaZulu-Natal, that are of importance in dermatological use, reported how various medicinal plants were grown more often by women than men at their homesteads. Also, if Western medicinal facilities were accessible, the traditional South Africans continue to be subject to indigenous medicinal plants. An ill person with a medical condition is taken to a Western doctor as an alternative or last choice for care when an indigenous intervention has failed (**ZOBOLO and MKHABELA, 2006**). All the women that were interviewed had a broad understanding of the traditional Zulu nomenclature and naming of plants. Their applications in traditional healthcare, and their health care treatment combinations for a wide range of conditions include stomach-ache, coughs, tooth-aches, headache, asthma, diarrhoea, wounds and diabetes (**ZOBOLO and MKHABELA, 2006**). Modern allopathic medicine is rooted deeply in primeval prescriptions and medications; and today it is still expected that many essential novel medications will be commercialized when discovered in the imminent forthcoming years by ensuing the principals provided through indigenous information and experiences (**VAN WYK et al., 2009**).

### **1.2.1 Global Context**

The WHO Reports (**2000; 2002; 2008**) on indigenous health care systems of medicine are adaptive and dynamic even though firmly rooted on past traditions. This vigour advocates that with suitable scientific recognition, support and basic traditional health care, traditional

medicinal systems will endure well into the next Century. Plants were once the only source of medicines for primary health care, however for many communities across the world they continue to provide mankind with new remedies Herbal medicine is an important element in most indigenous populations and in their traditional medicine systems. Almost 50% of allopathic drugs in clinical use worldwide are represented from plant derivatives and from natural plant products. While it is also known that 25% of allopathic drugs in medical applications are derived from higher plants (**KINGHORN and BALANDRIN, 1993**). Well known plant medicines include quinine (*Cinchona pubescens*), an alkaloid from the stem bark, which serves as an effective remedy for malaria; morphine, an alkaloid converted to codeine (*Papaver somniferum*), acting as a powerful analgesic is used to treat intense pain; with a world consumption of about 200 metric tons per annum (**HOGSHIRE, 2009**). Aspirin, atropine, reserpine and cocaine have all been sourced from plants (**ALI and AZHAR, 2000**). Plants are known as a source of essential new anticancer drugs for instance vincristine (*Catharanthus roseus*) and taxol (*Taxus* spp), a diterpenoid from leaves. Numerous plant constituents are currently manufactured for commercial use in large pharmaceutical laboratories. Examples include digitalis glycosides (a heart regulator drug), vincristine (an antitumor drug) and ephedrine (a bronchodilator drug used to decrease respiratory congestion); all of which were discovered through research on plants. Widespread and various international studies conducted in countries like India, (**LODHI et al., 2006; KOKANE et al., 2009**), Jordan (**KHALIL et al., 2007a; 2007b**) and Malaysia (**ABDULLAH et al., 2009**) on medicinal plants provide information on wound healing and antimicrobial activity. Despite the plethora of newly discovered secondary metabolites from microorganisms, allopathic medicines are developing widespread resistance and emerging microbial diseases are still on the increase in many nations of the world (**OKEKE et al., 2005**).

### **1.2.2 South African Context**

A rich cultural diversity within South Africa is revealed in the formal and informal primary health care systems of remedies that are currently adept in different parts of the country. The natural, practical and verbal transmission from generation to generation of traditional therapeutic health systems of the Xhosa/Sotho and Zulu language people, and the Nguni and Khoi San tribes have not been adequately recorded nor standardised and are purely distributed by word of mouth from one generation to another from its early origins (**HUTCHINGS, 1989; HUTCHINGS and VAN STADEN, 1994; HUTCHINGS et al., 1996**). The official systems of medicine are well recognized and organized and have only been introduced into the country

over the last 300 years by European settlers in South Africa and are exemplified by modern Western medicine or allopathic medicines. South Africa's rich diversity now incorporates the practice of a wide range of medicinal practices including those that have migrated into South Africa from Ayurvedic of Indian and Chinese homeopathic origin. Each medicinal system is an art and science that maintain health in its holistic sense of psychological, spiritual, social and physical aspects. For each culture solutions for health care that were found are in synchrony with the all-inclusive view of that culture. Doctors may make a diagnosis of a disease in terms of a causative pathogen and effectively treat it with antibiotics while a traditional healer may seek to understand the reasons behind the illness and treat the observed cause in addition to detailed treatments for the signs and indications of the condition (**HUTCHINGS, 1989; HUTCHINGS and VAN STADEN, 1994; HUTCHINGS et al., 1996**).

South Africa has a projected 200 000 recognized indigenous traditional healers in practice and almost 60% of South Africans consulted these healers whilst simultaneously using contemporary allopathic and biomedical services (**VAN WYK et al., 2009**). An extensive number of daily used medicines are extracts of plants; harvested in large quantities and traded in the sought-after and informal markets. South Africa's input to the international medicines market includes buchu (*Agathosma betulina*), the Cape aloes (*Aloe ferox*) and devil's claw (*Harpagophytum procumbens*) but indigenous counterparts exist for many of the well-known remedies. There is emergent awareness in regular and indigenous treatments as a basis for many new commercial health products (**GRIERSON and AFOLAYAN, 1999a; 1999b; McGAW et al., 2000; AFOLAYAN et al., 2002; LATEGAN et al., 2009**). South Africa has developed through the Medical Research Council's (MRC) Indigenous Knowledge Systems (IKS) Lead Programmes that redresses health traditions which has been a neglected health research priority in the country with intentions to be competitive globally. In South Africa, significant antimicrobial accomplishments continue to be reported from various medicinal plants (**GRIERSON and AFOLAYAN, 1999a; 1999b; McGAW et al., 2000; AFOLAYAN et al., 2002; LATEGAN et al., 2009**). The traditional people of the Eastern Cape Province practice various wound causing cultural activities like skin cuts on the face and body. Circumcisions are an annual event, resulting in a notably high rate of mortality due to severe infections of the wounds (**DILIKA et al., 1996**).

### **1.2.3 KwaZulu-Natal Context**

The extensive use of medicinal plants by the indigenous population in KwaZulu-Natal (KZN) Province, is the Province with the second largest population of 10.69 million (19.8%). The

African population in South Africa constitutes 80% (43.3 million) of the total population (**CENTRAL STATISTICAL SERVICES, 2015/16**) with an estimated population growth of 2.4% per year. According to reports, in excess of 400 plants are vigorously merchandised at muthi markets throughout KZN (**CUNNINGHAM, 1988; 1993; McKEAN, 1993**). Due to the fragmented distribution, topography and climate in KZN, a large spatial population of consumers concentrate in urban centres resulting in the need to access more remote plant populations as local numbers diminish due to indiscriminate gathering and socio-economic factors that sustain and stimulate demand (**MANDER, 1997; 1998**).

A survey conducted in 1995 reported that about 53% of sick African patients visited ethnic traditional healers once, with others having an average of three visits per year in Durban, KZN (**MANDER, 1998**). This lead to an estimation of the overall figure of black African patients in Durban to be around six million (**MANDER, 1998**). Traders in Durban are projected to trade approximately 950 tonnes per year with approximately 50% of medicinal plants acquired directly from plant collectors and the remaining from street traders. Other than a growing resident demand, there are suggestions of a fast growing demand from South Africa's neighbouring countries particularly Namibia and Botswana (**MANDER, 1997; 1998**). *Merwillia plumbea* and *Haworthia limifolia* are of the nine popular and most harvested plants in KZN (**MANDER, 1997**). In addition a further 1000 plants are used for traditional medical purposes in KZN and approximately 500 species are sold in extensive volumes at local markets. The bioactive ingredients and plant extracts in plant parts like the bark, leaves, rhizomes and roots are used in various combinations and while one part may be harmful another could be quite harmless. Whole plants are often harvested but seldom used for medicinal purposes. Leaves, stems/rhizomes and the underground parts like bulbs, roots and tubers are commonly used.

### 1.3 Medicinal Plants Used in this Study

The first research step was the selection of the eleven plants species which were collected based on ethnobotanical information; ethnodirected selection, including ethnobotanical and ethnopharmacological approaches and information about the traditional medicinal use of these plants to treat dermatological disorders and diseases specifically. The plants were also selected based on verifying the use of many species that are used by local traditional healers. Many of the selected plants exhibit an array of medicinal uses, of which all have a common thread of being identified by traditional healers as used for skin diseases and the plants were identified by a horticulturalist at the Silverglen Nursery, Chatsworth, KZN.

**Table 1.1** The selected plants that are used for wound healing purposes, for treating skin diseases and skin conditions

Plant species/ Family	Plant parts used	Voucher number	Medicinal uses
<i>Aloe arborescens</i> Mill. Xanthorrhoeaceae	Leaves, bulbs	SG01/UKZN	Leaves and bulbs are used for the management of HIV/AIDS, asthma, tuberculosis, gastrointestinal ailments, constipation, colds and fever, oesophageal cancer, stomach ache, burn wounds, abrasions, bruises; has significant wound healing, antibacterial, anti-ulcer, anti-inflammatory, anti-carcinogenic, hypoglycaemic properties, alopecic properties and purgative effects; inhibits cell proliferation and is used during childbirth ( <b>HUTCHINGS et al., 1996; KLOS et al., 2009; VAN WYK et al., 2009; CROUCH et al., 2006</b> ).
<i>Aloe aristata</i> Haw. Xanthorrhoeaceae	Leaves	SG02/UKZN	Leaf juices are used to treat chronic and severe dermatitis, wounds, burns, cuts, eczema, sunburn, insect stings, poison ivy skin irritations, abrasions and numerous dermatological conditions. Sap eases pain and reduces inflammation, inhibits cell proliferation and promotes growth of skin and connective tissue ( <b>JIA et al., 2008; BEDINI et al., 2009; VAN WYK et al., 2009</b> ).

<i>Aloe ferox</i> Mill. Xanthorrhoeaceae	Leaves	SG03/UKZN	Leaf gel is used for burns, wounds and various skin conditions like eczema and ringworm; arthritis, conjunctivitis, venereal sores, bruises, pimples, blisters, ringworm, boils ( <b>DIEDERICHS et al., 2009; VAN WYK et al., 2009</b> ). This group of plants is known to be excessively harvested for over 250 years ( <b>NEWTON and VAUGHAN, 1996; VAN WYK and SMITH, 2004</b> ).
<i>Bulbine frutescens</i> (L.) Willd. Xanthorrhoeaceae	Leaves, stems, roots	SG04/UKZN	Leaf sap is used for burns, rashes, blisters, insect bites, cracked lips, prickly heat pimples, acne, cold sores, mouth ulcers, cracked skin. Popular for skin and wound conditions, fever blisters, rashes, eczema and ringworm, and is antibacterial ( <b>DIEDERICHS et al., 2009; VAN WYK et al., 2009</b> ).
<i>Bulbine natalensis</i> Baker. Xanthorrhoeaceae	Leaves, bulbs, roots	SG05/UKZN	Leaf sap is directly smeared onto the skin to treat wounds, sores, burns, rashes, itches, ringworm, cracked lips and herpes, rheumatism, sunburn, mouth ulcers ( <b>VAN WYK et al., 2009</b> ).
<i>Eucomis autumnalis</i> (Mill.) Chitt. Asparagaceae	Stems, leaves, bulbs	SG06/UKZN	Bulb used for post-operative recovery, assist in healing fractures, emetics, treat fever, STI's, facilitate childbirth. Backache, inflammation, pain, rheumatism ( <b>DIEDERICHS et al., 2009; VAN WYK et al., 2009</b> ).
<i>Haworthia limifolia</i> Marloth. Xanthorrhoeaceae	Stems, leaves, roots	SG07/UKZN	Leaves are used to treat stomach complaints, sores, superficial burns, sun burn, purify blood, promotes fertility in women and cleans the digestive system. Enemas for internal tumours, sprains, fractures, boils, antischisomal and anthelmintic activity, antiseptic, anti-inflammatory properties ( <b>DIEDERICHS et al., 2009; VAN WYK et al., 2009; COOPOOSAMY and NAIDOO, 2011a</b> ).
<i>Hypericum aethiopicum</i> Thunb. Hypericaceae	Leaves, roots, stems	SG08/UKZN	Leaf oil extracts are used as a cure for wounds and first degree burns, treat backache and loin pain as well as fevers ( <b>ROOD 1994; BRUNETON 1995; HUTCHINGS et al., 1996; VAN WYK et al., 2009</b> ).
<i>Merwillia plumbea</i> (Lindl.) Speta. Asparagaceae	Roots, leaves, bulbs	SG09/UKZN	Bulb decoctions used to treat boils, sores, wound healing and sprains; also get rid of scar tissue, emetic for detoxifying and revitalizing the body. Various skin ailments, sprains, fractures, infertility, male libido, internal tumours, anti-inflammatory, antiseptic ( <b>CROUCH et al., 2006; DIEDERICHS et al., 2009; VAN WYK et al., 2009</b> ).



<i>Tetradenia riparia</i> (Hochst.) Codd. Lamiaceae	Leaves, stems	SG10/UKZN	Leaves used for colds, influenza, fever, malaria, swollen legs, headaches, chest complaints, diarrhoea, flatulence, nausea, respiratory problems, mouth ulcers, coughs, stomach ache and diarrhoea ( <b>WATT and BREYER-BRANDWIJK, 1962; DYSON et al., 1998; COOPOOSAMY and NAIDOO, 2011b</b> ).
<i>Zantedeschia aethiopica</i> (L.) Spreng. Araceae	Stems, bulbs/rhizomes	SG11/UKZN	Leaves soothes burns, insect bites, draws wounds, sores and boils; boiled rhizomes for bronchitis, asthma, heartburn, rheumatism; gargles for mouthwash. Treat wounds, inflammation, sores, boils, rheumatism, gout, bronchitis, asthma, heartburn, sore throat ( <b>DYSON et al., 1998; VAN WYK et al., 2009</b> ).

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## 1.4 Dermatological Significance of Skin and Health

The skin is the human's largest organ and is essential for good health. Skin diseases occur in several forms and are categorised as non-contagious and contagious conditions; the principal reasons include a range of microorganisms like the bacterial, parasitic and fungal diseases. These are widespread mostly in the coastal and rural environments (DAVIS et al., 1980). SATO et al. (2013) found that ethanol extracts of some Chinese medicinal plants used *in vivo* resulted in reducing acne and dry skin (serosis) and had a protective role in forming a barrier on the skin. In India, NAGORI and SOLANKI (2011) reported on a number of plants and their role in wound healing. Plants displaying dermatological abilities are highly pursued for their ability to stop blood loss, increase healing and appease skin unprotected from burns and breakage (LEWIS and ELVIN-LEWIS, 1977). Studies on HIV-positive patients who seek medical treatment have a high frequency of skin diseases and it is advised that skin disease specialists be incorporated into their preliminary assessment and continuing long term health care. Skin conditions are known to occur in HIV immune-compromised patients in sub-Saharan Africa and a large percentage of skin diseases are found amongst those populations living in the rural communities of South Africa. Some of the common skin conditions associated with HIV/AIDS include those caused by *Herpes* viruses, *Molluscum contagiosum*, oral hairy leucoplakia Kaposi sarcoma, photodermatitis, thrush and *Prurigo nodularis* (NAAFS, 2004).

## 1.5 The Management of Wound Healing and Skin Diseases

Wounds and skin diseases are the most important causes of physical disabilities and mortality. Wound healing is a natural progression that commences with trauma and concludes with scar formation. According to the Wound Healing and Dermatological Societies, physical injury to the skin causes susceptibility to infection in both the anatomy and physiology of a human body (STRODTBECK, 2001). The goals of treating wounds and skin diseases are decreasing the risk factors that obstruct healing, increase the healing process and decrease the frequency of wound infections. Numerous medicinal plants are useful all over the world due to their therapeutic compounds. Wounds and skin diseases epitomize an important affliction worldwide. Nosocomial and hospital-related and facility-care-related infections are on the increase (WEINSTEIN, 1998; 1991). Many skin infections are frequent in patients with poor nutrition and gastro-intestinal

infections exacerbated the problem, especially in unhygienic conditions and overpopulated communities. **KUMAR et al. (2007b)** reported that almost six million people worldwide suffer from chronic wounds and skin conditions. A study conducted in the Eastern Cape Province of South Africa, reported the importance of plant extracts for wound healing from circumcision rituals by the Xhosa people. The wounds were dressed and bandaged with a mashed plant material of *Helichrysum* species and *Boophane disticha* and were successful in the wound healing process (**DILIKA et al., 1996**). Traditional circumcision carries a high risk for infection and susceptibility to common bacteria which infect wounds, namely *Staphylococcus aureus*, *Streptococcus* species and *Escherichia coli*. The leaves of the plants listed in Table 1.1 exhibited the highest antimicrobial activity. In a study on *Funtumia*, *Raphyostylis*, *Butyrospermum*, *Serataria*, *Parkia* and *Curculigo* species used in the management of wound and skin diseases, the findings reported a high activity index for antimicrobial and antioxidant assays from various metabolite products with regards to the effects of the plant extracts for skin diseases (**ADEBAYO-TAYO et al., 2010**). In other studies, **WADANKAR et al. (2011)** and **BHAT et al. (2012)** screened the usage of Indian plants for treatment of skin disorders and wounds in a study conducted in Kannada District. It was found that 106 plants used in herbal medications for wounds and skin were based on indigenous knowledge. In the study conducted in Washim District, Maharashtra 39 plants were identified with wound healing properties. A study in South Africa (**COOPOOSAMY and NAIDOO, 2011a; 2011b**) reported the antimicrobial use of *Tetradenia riparia* for common skin conditions like sores and wounds that could be used for secondary infections in HIV/AIDS infected patients.

## **1.6 The Role of Plants in Wound Healing**

The human skin performs numerous vital functions primarily forming a barrier of protection against infection. When the protective wall is damaged infectious microorganisms have direct admittance into the skin and penetrate the body. Cutaneous wound repair is a series of events including inflammation, proliferation, maturation and remodelling. The normal skin anatomy and function when disturbed due to an opening, rupture or break of the skin surfaces which may be due to physical injuries are known as wounds (**STRODTBECK, 2001**).

According to **KUMAR et al. (2007b)**, the burden of chronic wounds is recorded in nearly six million sufferers worldwide. Wounds are an ideal substrate for infection and prolonged recovery

may have consequences on other organs of the body (**ROBERTS et al., 1998**). Wound conditions are the most frequent motive for compromised wound healing due to bacteria such as beta-haemolytic *Streptococcus* Group A, *S. pyogenes*, *S. aureus*, *E. coli*, *Corynebacterium* species, *E. faecalis*, *P. aeruginosa*, *K. pneumonia* and *Acinetobacter baumannii* (**RODGERS et al., 2000; CHURCH et al., 2006; KUMAR et al., 2006**).

Many medicinal plants registered as pharmaceutical drugs (**AKERELE, 1988; CUNNINGHAM, 1988; FARNSWORTH, 1994; AFOLAYAN and MEYER, 1995; 1997; KLOS et al., 2009; COOPOOSAMY, 2011; NAGORI and SOLANKI, 2011**). Although there are many wound healing medicinal plants and bioactive compounds, characteristic events and chemical properties have not yet been adequately assessed; a knowledge of these could contribute to their traditional worth in skin healing management (**GOLDSTEIN et al., 1980; 1994; SAITO et al., 1982; PUSZTAI, 1991; IMANISHI, 1993; COOPOOSAMY, 2010; STREET and PRINSLOO, 2013**). An example of a patented medication from traditional medicinal plants used for wound healing is that of a topical cream comprising a combination of *Plectranthus amboinicus* and *Centella asiatica* extract with salvigenin and asiaticoside. These two components promote wound healing in diabetic patients with foot ulcers (**NAGORI and SOLANKI, 2011**).

## **1.7 Significance and Aims of the Study**

A number of organizations were formed by the contributions of the African Union Decade of Traditional Medicines (**AU, 2005**), together with the World Health Organisation (**WHO, 2002; 2008**), and the Biodiversity Convention (**DE SOUZA DIAS, 2016**) in order to promote and regulate the use of traditional medicinal plants worldwide. These advocate the advancement of indigenous remedies through the progress in medicinal plants research, indigenous knowledge preservation and sustainable applications in public health care (**HEINRICH et al., 2004**). A major concern of the Council for Medicines Control in South Africa was the lack of information on certifiable outcomes and statistics of traditional medicines from surveillance and knowledge systems. Models and practical data regarding the procedures for efficacy, safety and quality of the plant extracts to sufficiently safeguard public health do exist (**SCOTT et al., 2004**). Indigenous healers are respected as main and supplementary therapeutic plant investigators for their expertise and experience of the medicinal usage of these plants (**TIMMERMANS, 2003**).

Research conducted in South Africa on indigenous plants medicines are antimicrobial and effective against pathogenic skin Gram-positive bacteria, Gram-negative bacteria and fungi. These include various medicinal plant species that are known to be antimicrobial and used for healing cold sores, insect bites, rashes, burns, boils, cracked skin and mouth blisters (**RAINA et al., 2008; NAGORI and SOLANKI, 2011**). A minimal availability of resources and information on remedies precisely for the management of wounds and skin diseases is of concern (**LALL and KISHORE, 2014**). Dermatological studies are looked upon as a speciality that has equivalent medicinal and invasive characteristics known to treat illnesses broadly including cosmetic skin problems as well as conditions of the nails, scalp and hair. Carbuncles, boils and sores are related skin ailments with consequences of wounds that occur on the skin. Pathogenic infections are the leading challenge in the wound healing progression process of skin diseases (**LALL and KISHORE, 2014**). The availability of cost effective and available access to wound and skin healing preparations and an evaluation of medicinal plants commonly used routinely for the management of skin disorders is of indispensable significance.

Some of the plants included in this study namely *Aloe aristata*, *Aloe arborescens*, *Aloe ferox*, *Bulbine frutescens*, *Bulbine natalensis*, *Merwillia plumbea*, *Haworthia limifolia*, *Hypericum aethiopicum*, *Zantedeschia aethiopica*, *Tetradenia riparia* and *Eucomis autumnalis* are on the Red Data List due to their high demand, over-exploitation and many of them are threatened with extinction (**MANDER, 1997; 1998**). The plants were collected from the Silverglen Conservancy Nursery in Durban, KZN for investigation and validated for their use in skin conditions and wound healing.

The application of many plants for wound healing and dermatological diseases are crucial to unravelling the responses to questions around contesting challenging skin diseases when obstruction of pathogens by medications and allopathic treatment endures and resistance increases over time. The aim of this study was to conduct various ethnopharmacological assays on the extracts of 11 South African indigenous therapeutic plants that are commonly used in the management of skin-related conditions and wounds. These were measured for their management of antimicrobial activity, their effects found regularly associated with indications occurring in purulent sores, boils and wounds as well as an evaluation of their anti-inflammatory activity,

cytotoxicity effects and phenolic content was investigated. To achieve this, the purpose of the study was to:

- Determine the antimicrobial activity of selected plant extracted with different solvents;
- Evaluate the plant extracts for anti-inflammatory activity (Lipoxygenase and Nitrogen oxygenase assays);
- Analyse the phenolic composition of the plant species;
- Evaluate the cytotoxicity effects (Brine shrimp and MTT assays) of the extracts;
- Determine the bioactivity of the plant extracts in various antioxidant assays (DPPH, FRAP, CLAMS); and
- Evaluate the wound healing properties of the selected plants (protein binding capacity and wound healing assay).

# CHAPTER TWO

## ANTIMICROBIAL ACTIVITY

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### 2.1 Introduction

The knowledge of wound healing by many traditional physicians predominantly in countries like China and India holds treasured evidence for treating skin diseases, burns and wounds (BHARADWAJ and GAKHAR, 2005; KUMAR et al., 2007b; AYYANAR and IGNACIMUTHU, 2009; PATIL et al., 2009; KUVAR and BAPAT, 2010; SUBRAMANIAN et al., 2011). South African studies on wound healing plants are limited and further investigations are required (WIDGEROW et al., 2000; PATHER et al., 2011; VAN WYK, 2011). According to LIGHT et al. (2005), minimum information is available on the extensive number of plants used with wound healing capacities in South African plants. A survey taken at the Umlazi and Durban muthi markets in KwaZulu-Natal with traditional health practitioners resulted in a biological evaluation of a number of medicinal plants applied for wound healing and skin condition therapies (COOPOSAMY and NAIDOO, 2011b). The increase in studies on antimicrobial activity has demonstrated the increased global interest in medicinal plants and their antimicrobial potential (GHUMAN and COOPOOSAMY, 2011). Traditional populations of South Africa have used a variety of plants to treat infections and topical wounds (RABE and VAN STADEN, 1997; MULHOLLAND et al., 2016). Infectious diseases related with painful wounds, cuts and burns, and animal bites, or the permeation of foreign instruments indicate the presence of pathogens. Wounds and infections are frequently infected with a variety of disease-causing microorganisms related to the skin including anaerobic pathogens. *Staphylococcus aureus* is one common pathogen associated with wounds that are infected and have a pus-filled discharge (MADIGAN et al., 2015).

Antimicrobial properties of the plants used in this study were tested against common bacteria infecting wounds, viz. *Staphylococcus aureus*, *Streptococcus pyogenes* and *Escherichia coli*. Wounds particularly from burns are also a susceptible site for opportunistic colonization by microorganisms and may lead to high mortality and morbidity. A study on wound stab cultures of bacteria in burn patients reported *Pseudomonas aeruginosa* as the organism most frequently

isolated (54%) (**ARSLAN et al., 1999**). Some of the bacteria isolated from burn wounds includes *Proteus mirabilis*, *Klebsiella pneumonia*, *Escherichia coli* and various *Pseudomonas* species (**ARSLAN et al., 1999; BAGDONAS et al., 2003**). In addition, wounds are commonly infected with fungal pathogens like *Candida* species. Infectious diseases are aggravated further by elements like insufficient public health practices, reduced hygiene and congested living circumstances (**KERR and LACEY, 1995**).

## **2.2 Microbial Infections**

### **2.2.1 Bacterial Infections**

Many studies describe the following causative microorganisms: *Klebsiella* species, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus* species, *Escherichia coli* and *Candida* species being present in 11-31% of all burn wounds (**BAGDONAS et al., 2003**). The most common causative agent is *Staphylococcus aureus*, relatively pathogenic to humans and is found on the skin and mucosa of up to 40% of the population. Burn injuries are often infected in 30% of cases with *Staphylococcus* species of which three are important in skin conditions; *S. epidermidis*, *Staphylococcus aureus* and *Staphylococcus saprophyticus*. (**BAGDONAS et al., 2003**). The antibiotic resistance of these strains remains a global problem.

### **2.2.2 Fungal Infections**

Fungi usually invade habitats in the damp parts of the human body where skin fasciae meet; these include areas that are in the genital area, between the toes and under the breasts. Frequently occurring fungal skin infections are caused by yeasts (*Candida*) or dermatophytes, such as *Epidermophyton*, *Microsporum* and *Trichophyton*. Many of these fungi reside in the uppermost stratum of the epidermis (stratum corneum) and do not infiltrate deeper (**VAN HEES and NAAFS, 2009**). Obese and older individuals are more susceptible to these infections for one important reason, that they have areas in the body with many skinfolds. Diabetics have a tendency to be more vulnerable to fungal infections as well. Particularly important to note, is that fungal infections on one part of the body spread causing rashes on other uninfected parts of the body. A foot or fungal infection between the toes may cause bumpy and itchy rashes on the fingers (**VAN HEES and NAAFS, 2009**). Skin eruptions are allergic responses due to the fungus and are caused by touching the infected area. Physicians usually confirm fungal infections through symptoms of red, irritated,



or scaly rashes in one of the commonly affected areas and through diagnostics like the microscopic examination of scrapings from a small amount of skin or cultures grown in medium where the specific fungus can be identified. Almost 80% of all fungal infections are caused by *Candida albicans* (VAN HEES and NAAFS, 2009). *C. albicans* commonly lives on the mucosal and skin surfaces, any modifications in the host's environment will lead to its propagation and ensuing skin disease. A severe skin infection, candidal vulvovaginitis of the perineum in women is caused by a fungus; the symptoms are characterized by reddish, scaly itchy skin and mucosa, marginal pustules and a creamy discharge. The equivalent in uncircumcised men is an infection called balanitis, characterized by shiny reddish plaques on the glans penis also affecting the scrotum (VAN HEES and NAAFS, 2009). Skin infections caused by *Candida* occur on just about any part of the mammalian body, but are more frequently occurring in areas where skin may touch or rub together like the skin folds, groin, armpits and the skin found between the toes and fingers. The fungus flourishes in environments that are warm, sweaty and moist (VAN HEES and NAAFS, 2009).

### 2.3 Antibiotic Treatment of Skin and Wound Infections and Diseases

Antibiotics are toxic compounds that are naturally formed by fungi and bacteria to eliminate competing microorganisms. The first antibiotic activity was discovered when anthrax bacteria were isolated and identified (JOHNSON, 2009). Antibiotics are natural products that differ from growth factor analogs which are artificial compounds. Historically, the most important antibiotic group is the  $\beta$ -lactams which include penicillin, cephalosporin and cephamycins. They share a distinguishing structural constituent, the  $\beta$ -lactam ring. Penicillin G was the first isolated  $\beta$ -lactam antibiotic; it is ineffective against Gram-negative bacteria as their cell walls are resistant to the antibiotic but are effective against Gram-positive bacteria (MADIGAN et al., 2015). Selectively antibiotics attack microorganisms short of harming the host cells by either one of two mechanisms. These mechanisms are the inhibition of cell wall synthesis through bactericidal antibiotics like penicillin and cephalosporin and damaging the cell wall by increasing permeability resulting in leaking of the cell contents causing cellular death. Mammalian cells lack cell walls and have membranous outer coverings, therefore antibiotics are unable to damage these cells. Those antibiotics that prevent bacterial growth, for example tetracycline and erythromycin, inhibit DNA and RNA formation or inhibit protein synthesis by ribosomes (MADIGAN et al., 2015).

Bacterial infections like meningitis and typhoid fever were commonly responsible for high death rates. Antibiotics have reduced the deaths of millions of people with minimal side effects (JOHNSON, 2009).

Penicillin and erythromycin are widely used antibiotic drugs against those microbes which mutate easily. (JOHNSON, 2009). Prescription medications acquired over-the-counter, like clotrimazole or ketoconazole, belonging to a class of anti-fungal drugs called the azoles were found not to have debilitating effects on the patient when associated with other anti-fungal drugs like amphotericin B or nystatin. If the infection persists or becomes more severe, treatment will depend on the locus and severity of infection (MTHETHWA and DE WET, 2011). *Trichophyton* species causing tinea pedis, may be managed effectively with anti-fungal ointments such as terbinafine hydrochloride, butenafine hydrochloride, tolnaftate (a synthetic thiocarbamate), miconazole nitrate, clotrimazole and undecylenic acid. Severe, complicated infections use oral ketoconazole effectively in the management of *T. rubrum* infections, however its use is no longer advised resulting in liver damage. Oral terbinafine, itraconazole or fluconazole have all been shown to be safer, effective treatments (MTHETHWA and DE WET, 2011). Topical anti-fungal creams like terbinafine and naftifine are effectively used against tinea caused by *T. rubrum*. *Trichophyton rubrum* infections have been found to be vulnerable to photodynamic management like laser irradiation and photoactivation. *Proteus mirabilis* is susceptible to many antibiotics except tetracycline while 20% of *P. mirabilis* strains are unaffected by first-generation cephalosporins and ampicillins (MTHETHWA and DE WET, 2011).

Protein synthesis inhibitory antibiotics may cause side effects due to the similarity of human and animal ribosomes to bacterial ribosomes (JOHNSON, 2009). Classification of antibiotics is based on the type of bacteria affected. Gram-positive bacterial cell walls have a dense outer covering made up of peptidoglycan whilst bacteria that are Gram-negative are known to have a thinner stratum made up of peptidoglycan together with an inner and outer membranous layer. The structural variations in bacteria cell walls are significant for the effective antimicrobial action of antibiotics (JOHNSON, 2009).

## 2.4 Materials and Methods

### 2.4.1 Plant Identification, Selection and Collection

The identification, selection and collection of medicinal plants was based on their reported traditional uses and demand in treating wounds in the Province of KwaZulu-Natal. The collection of fresh samples allowed immediate processing of fresh plant material parts prior to extraction. All eleven of the plant species were available at, and collected from, the Silverglen Plant Conservancy, situated in Chatsworth, Durban, South Africa. Voucher specimens were prepared and verified by an experienced horticulturalist at the conservancy (Table 1.1). The voucher specimens were placed for storage at the John Bews Herbarium, University of KwaZulu-Natal (UKZN) in Pietermaritzburg. The medicinal materials were divided into component parts namely the leaves, roots and bulbs/corms prior to preparations made for drying in an oven at 50 °C for five days and were thereafter pulverized into fine particles using a Wareing Commercial blender and put in storage at room temperature in airtight glass bottles (NCUBE et al., 2011).

### 2.4.2 Plant Extract Preparation

A selection of five solvents (methanol, hexane, acetone, chloroform and dichloromethane) were each used to extract 20 g powdered ground samples of plant material. This process was carried out using a sonication bath with ice for an hour together with 20 ml/g (w/v) of extractant. Effective procedural determination of biologically active composites from plant materials depended on the type of solvent used in the extraction technique. The five solvents were selected based on varying polarity; these included non-polar hexane, chloroform, dichloromethane to more polar acetone and methanol. The crude plant extracts were clarified using a vacuum and filtered in a Büchner funnel through Whatman No. 1 filter paper and the extracts were then concentrated *in vacuo* at 35 °C with the aid of a rotary evaporator (Rotavapor-R, Büchi, Switzerland). All of the concentrated extracts were then dried at room temperature using a fan and then stored at 4 °C. These were stored for further use in the various assays that were performed in triplicate (ELOFF, 1998a; 1998b). The percentage yield of plant extract from the extracting solvents were calculated as the proportion of the dried extract mass to the mass of the ground plant sample. The plant extracts were subjected to two types of *in vitro* antimicrobial analysis conducted in triplicate.

## **2.5 Antimicrobial Activity Assays**

### **2.5.1 Microbial Stock Culture Preparation**

Fourteen stock strains of bacteria were used in the bioassay. These were cultured, then incubated overnight in McCartney bottles with 10 ml Mueller-Hinton (MH) broth at 37 °C. Thereafter the cultures were streaked onto Mueller-Hinton agar (Merck, Germany). Sterile (25 ml) MH agar was dispensed into plastic plates or petri dishes and permitted to gel at room temperature and cooled at 4 °C overnight. The plates were streaked with stock strains of bacteria and sub-cultured. These bacterial-inoculated plates were placed into an incubator at 37 °C for 24 h and bacterial colonies allowed to grow. The plates were then stored at 4 °C to prevent overgrowth of bacterial colonies. The stocks were sub-cultured following the same procedure once a month in order to maintain viability. A similar procedure was followed for the maintenance of the four fungal strains with Yeast Malt (YM) broth and agar (Becton Dickinson, USA) used in place of MH broth and agar.

### **2.5.2 Antibacterial Activity**

Hexane, chloroform, dichloromethane, acetone and methanol were the five extract solvents used for each of the eleven plant species to achieve preliminary evaluation using the disc-diffusion method for antibacterial activity (YU et al., 2003).

#### **2.5.2.1 Bacterial Disc Diffusion Assay**

Five extracts from each of the eleven plants species were first evaluated using the disc diffusion method. The obtained plant extracts were tested for antimicrobial activity by using two mg/ml of each extract. Fifty microliters of each extract were transferred on each of three sterile six mm discs of Whatman No. 1 filter paper. Plant extracts were soaked in ethanol with water (95% v/v). The extracts obtained were evaporated to syrupy residues (ELOFF, 1998a) which were dried. The positive control used in the assay was streptomycin. Preparation of the stock solutions were carried out by preparing and dissolving 100 mg of dry extract in 1 ml of ethanol and water separately to obtain a concentration of 100 mg/ml dilutions of the stock solutions. The stock solutions were then mixed with phosphate buffer at pH six to evaluate the antibacterial activity (ELOFF, 1998a). The antibacterial activity of these extract solutions was then tested on the selected microbial cultures. The test organisms (Table 2.1) were tested on the probability of them being present in patients suffering with wounds and superficial skin disorders. Plates having only Mueller-Hilton agar

functioned as the controls. Zones of inhibition were measured and those  $\geq 10$  mm were considered as good activity and were selected for the MIC experiment outlined below.

### **2.5.2.2 Antibacterial Microdilution Minimum Inhibitory Concentration Activity**

Those extracts that were observed to have zone inhibitions of  $\geq 10$  mm were further investigated against the various Gram-positive and Gram-negative strains of bacteria related to skin conditions. Twenty plant extracts were selected from this assay for screening using the minimum inhibitory concentration assay. The microplate dilution method was used to determine the minimum inhibitory concentration (MIC) values for the antibacterial activity of the plant extracts (**ELOFF, 1998a; 1998b**). Each resuspended extract (50 mg/ml) was selected and one hundred microliters was serially diluted two-fold with sterile distilled water in duplicate. A 96-well microtitre plate (Greiner Bio-one GmbH, Germany) was used for each of the fourteen strains of bacteria. Similarly a 0.1 mg/ml, two-fold serial dilution of neomycin (Sigma-Aldrich, Germany) was used as the positive control. Seventy percent ethanol and water were included as the negative/solvent controls. Mueller-Hinton (Oxoid, England) overnight broth cultures (incubated at 37 °C in a water bath with an orbital shaker) of the 14 strains of bacteria tested: seven Gram-negative strains and seven Gram-positive strains (Table 2) were mixed in sterile MH broth and diluted to give a final dilution of approximately  $10^6$  cfu/ml (colony forming units). The wells were dispensed with one hundred microliters of each bacterial culture. Parafilm was used to cover the plates and they were incubated at 37 °C. Growth of the bacteria was assisted by adding 50  $\mu$ l of 0.2 mg/ml *p*-iodonitrotetrazolium chloride (INT) (Sigma-Aldrich, Germany) and a supplementary incubation for a further 1 h at 37 °C. The colourless tetrazolium salt was biologically reduced to a red end product if there was a presence of active microorganisms and the MIC values were logged. The MIC values were also recorded for the concentrations in the last wells in which no colour was observed after adding the INT indicator. Growth of bacteria in the wells was observed by a reddish-pink colour. Triplicate measurements were recorded for the MIC assay. Values were also recorded for the lowest concentration resulting in complete inhibition of bacterial growth.

Of the medicinal plants selected, extracts from twenty plants yielded inhibitions of  $\geq 10$  mm. The minimum inhibitory concentrations (MIC) were determined in more detail using the microdilution bioassay against the seven Gram-negative and the seven Gram-positive strains of bacteria that are associated with skin conditions as defined by **ELOFF (1998a; 1998b)** and detailed in **NCUBE et**

**al. (2011).** Resuspension of all these extracts in 70% ethanol was done. A starting concentration of 0.1 mg/mL Neomycin (Sigma Aldrich, Germany) was used as a positive control for each bacterium. Water and ethanol (70%) was used as negative control and solvent. The negative control represented the growth of untreated organism during the test period. The addition of ethanol as a co-solvent to aid dissolution of the positive control and test sample had to be accounted for because ethanol could be toxic to microbes (**OJALA et al., 2000**). The negative control thus consisted of the bacterial suspension in the relevant broth and ethanol to correspond with the concentration of ethanol used to dissolve the test samples. The negative controls thus represented 100% viability for the microbes taking into account the corresponding concentration of ethanol that was used. The assay was carried out in triplicate per assay.

### **2.5.3 Antifungal Activity**

#### **2.5.3.1 Disc Diffusion Assay**

Preliminary screening using agar disc diffusion assays were conducted. Two mg/ml for each of the plant extracts were tested for antifungal activity. Fifty microliters of each extract was transferred on each of three sterile six mm discs made from Whatman No. 1 filter paper. These were used in phosphate buffer at a pH of six to determine the antifungal activity (**ELOFF, 1998b**). The solutions were tested for antifungal activity using the four fungal cultures using fluconazole as a positive control. The test organisms (Table 2.2) were selected on the knowledge of them being present on the skin and on wounds of patients. Plates with yeast malt agar were used as the controls. Zones of inhibition were measured and those  $\geq 10$  mm were considered as good activity and were selected for the MIC experiment.

#### **2.5.3.2 Antifungal Microdilution Minimum Inhibitory Concentration Activity**

Once disc-diffusion preliminary screening was completed, twenty extracts were selected for further assessments using the microplate dilution technique against four fungal strains (*Candida albicans* (ATCC 2670), *Candida tropicalis* (ATCC 66029), *Trichophyton mentagrophytes* (9533) and *Trichophyton rubrum* (28188) frequently related with skin conditions (Table 2.2). The MICs for the extracts were determined using the microdilution bioassay as described by **ELOFF (1998b)** and modified for fungi (**MASOKO et al., 2007**) as detailed in **NCUBE et al. (2011a; 2011b)**. All extracts were re-suspended in 70% ethanol and Amphotericin B (Sigma Aldrich, Germany) at a starting concentration of 2.5 mg/ml was used as a positive control against each fungus. A fungal

culture incubated overnight was grown in Yeast Malt (YM) broth. A quantity of four hundred microlitres of the overnight culture was added to 4 ml of sterile saline and the values read at an absorbance of 530 nm. Adjustments were made for the absorbance using sterile saline to an equivalent of a 0.5 M McFarland standard solution. This standardised fungal stock was used to make a 1:1000 dilution with sterile YM broth and a final inoculum of approximately  $10^6$  cfu/ml. Known concentrations of the dried crude plant extracts (PE, ethanol and DCM) were resuspended in 70% ethanol. A two-fold serial dilution of one hundred microlitres of each extract was made with sterile water in a 96-well microtitre plate. Similarly, a two-fold serial dilution of Amphotericin B (Sigma-Aldrich, Germany) (2.5 mg/ml) was prepared for use as the positive control while 70% ethanol and water were used as solvents and negative controls respectively. Each well then received one hundred microlitres of the dilute fungal culture. These plates were then covered with parafilm and incubated at 37 °C for 24 h. Thereafter an amount of 50 µl (0.2 mg/ml) INT was added and further incubated at 37 °C for 24 h. When the plate wells remained clear it served as an indication of the inhibition of fungal growth. The MIC values were recorded as the lowest concentrations that prevented fungal growth after 48 h. Fungicidal activity was determined by adding 50 µl of sterile YM broth to all the clear wells of the plates and extending the incubation at 37 °C for a further 24 h. Thereafter, the minimum fungicidal concentrations (MFC) were documented as the last clear wells detected. The assay was conducted in triplicate.

## 2.6 Description of Microorganisms

The fourteen bacterial strains were Gram-positive (seven) and Gram-negative (seven) are commonly found associated with skin conditions. These test organisms (Table 2.1 with ATCC numbers and infectious activity) were tested on the probability of them being present in patients suffering with wounds and superficial skin disorders. Four fungal strains (*Candida albicans* (ATCC 2670), *Candida tropicalis* (ATCC 66029), *Trichophyton mentagrophytes* (9533) and *Trichophyton rubrum* (28188) frequently related with skin conditions (Table 2.2 infectious activities).

**Table 2.1** Bacterial strains used for screening antibacterial activity in this study

Bacteria	Gram test	ATCC No.	Infectious activities
<i>Proteus vulgaris</i>	-	49132	Nosocomial infections and urinary tract sepsis infections (UTI's) caused by <i>Proteus</i> together with other bacteria like <i>Enterobacter</i> , <i>Pseudomonas</i> , <i>Klebsiella</i> , <i>Enterococcus</i> and <i>Staphylococcus</i> species (VAN HEES and NAAFS, 2009).
<i>Proteus mirabilis</i>	-	29245	Majority of <i>Proteus</i> infections cause wound infections, pneumonia, septicaemia and UTI's; these are commonly occurring in hospitalized patients (VAN HEES and NAAFS, 2009).
<i>Shigella sonnei</i>	-	9290	90% <i>Shigella</i> species invade the abdominal mucosa, cause inflammatory damage to the mucosal wall (VAN HEES and NAAFS, 2009).
<i>Klebsiella pneumoniae</i>	-	13882	Opportunistic pathogens, nosocomial infections, urinary and respiratory tract infections, intestinal to lethal septicaemia (WILLEY et al., 2011).
<i>Escherichia coli</i>	-	13706	Utero-toxigenic strain, urinary tract infection, gastro-intestinal diseases, haemorrhagic colitis (WILLEY et al., 2011).
<i>Pseudomonas aeruginosa</i>	-	9027	Deeper dermal abscesses (KUMAR et al., 2007a).
<i>Enterobacter aerogenes</i>	-	350029	Infections vary from the skin, eyes, soft tissues, UTI's, bacteria infiltrate the blood, lower respiratory tract, endocarditis, to the intra-abdominal area, septic arthritis, osteomyelitis and central nervous system (VAN HEES and NAAFS, 2009).



<i>Actinomyces brasiliensis</i>	+	16404	Infections occur in the oral cavity, eye, and tissues next to dental implantation areas, tooth extraction wounds cervicofacial, thoracic, abdominal actinomycoses, the female genital organs ( <b>VAN HEES and NAAFS, 2009</b> ).
<i>Micrococcus</i> species	+	700405	Skin microflora, recurrent bacteria in blood, endocarditis, meningitis, septic shock, arthritis and cavitating pneumonia ( <b>VAN HEES and NAAFS, 2009</b> ).
<i>Staphylococcus aureus</i>	+	29737	Superficial infections, skin and glands, mucous membranes, folliculitis, contagious impetigo, upper respiratory tract, pharynx, mouth, blood, mammary glands, intestines, potentially serious pathogen causing toxic shock syndrome ( <b>WILLEY et al., 2011</b> ).
<i>Streptococcus pneumoniae</i>	+	6301	Impetigo infectious blisters, pustules, oozing erosions and yellow crusts ( <b>KUMAR et al., 2007a</b> ).
<i>Streptococcus pyogenes</i>	+	12384	Skin, nose and mouth macules, lesions ( <b>KUMAR et al., 2007a</b> ).
<i>Bacillus subtilis</i>	+	6633	Diarrhoea, nausea, vomiting, powerful occupational allergen ( <b>WILLEY et al., 2011</b> ).
<i>Staphylococcus epidermidis</i>	+	12228	Infections of the lower urinary tract, common causes of skin infections such as pimples, boils, carbuncles, and abscesses ( <b>SINGER and TALAN, 2014</b> ).

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**Table 2.2** Fungal strains used for screening antifungal activity in this study

<b>Fungi</b>	<b>ATCC No.</b>	<b>Infections/diseases</b>
<i>Candida albicans</i>	2670	Candidiasis, thrush, infect the nails, skin, gastrointestinal tract, mucous membranes of oral and vulvo-genital areas, lips, corners of mouth, sores and systemic diseases ( <b>KUMAR et al., 2007a</b> ).
<i>Candida tropicalis</i>	66029	Candidiasis, mucous membranes, and gastrointestinal tract, infections of the skin, nails and systemic diseases ( <b>KUMAR et al., 2007a</b> ).
<i>Trichophyton rubrum</i>	28188	Dermatophytosis in keratinized tissue like skin, nails, hair, beard, scalp, causes athlete's foot, tinea, Athlete's foot, crotch itch and ringworm ( <b>KUMAR et al., 2007a</b> ).
<i>Trichophyton mentagrophytes</i>	9533	Tinea, athlete's foot, ringworm infections, feeds on the keratin in nails, hair and dead skin ( <b>KUMAR et al., 2007a</b> ).

## 2.7 Results and Discussion

Plant extracts from *A. ferox*, *A. arborescens*, *H. aethiopicum* and *E. autumnalis* were active against nearly all of the tested bacterial and fungal strains (Tables 2.3 and 2.4). All of the plant species exhibited some degree of antimicrobial activity. The observed antimicrobial efficacy demonstrated by some plant species on all the tested extracts provides significant and beneficial characteristics of these medicinal plant extracts in the treatment of skin diseases and wound healing. The two *Aloe* species and *H. aethiopicum* consistently exhibited significant antimicrobial activity and warrant further investigations and possible isolation of bioactive principles. Disc-diffusion assays are represented in Tables 2.3 and 2.4. Although the disc diffusion assay is still a valid technique, in general, it would not be recommended for anaerobic bacteria and fungi. Depending on the individual case to be investigated, it is recommended to use the disc diffusion assay for measuring the inhibition of a new drug. There are limitations to disc diffusion method which is why the method is done in triplicate to verify the results. Results can be unexpected or borderline. In such cases another method of testing may be required or the test may need to be repeated for confirmation. The possibility for some extracts to be inactive in the disc diffusion assay and active in the microdilution assay exists as the susceptibilities of the antibiotic vary. Susceptibility can vary even within a species also (with some strains being more resistant than others), antibiotic

susceptibility testing is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection *in vivo*.

The MIC's values for antibacterial and antifungal activity are presented in Tables 2.5 and 2.6 respectively. **RÍOS and RECIO (2005)** suggested that MIC values are of interest when MICs of 0.1 mg/mL and 0.01 mg/mL for extracts and isolated compounds are recorded. **FABRY et al. (1998)** defined active crude extracts as those having MIC values < 8 mg/mL. In this study, MIC values of less than 1 mg/mL were considered to have significant activity. Of the different extracts, chloroform extracts showed the highest activity for the majority of the tested extracts. Overall, many of the extracts were effective across a wide range of both Gram-positive and Gram-negative bacteria. *S. aureus* (Gram-positive) and *P. mirabilis* (Gram-negative) being the most susceptible strains. The chloroform and DCM extracts of *A. ferox* and *A. arborescens* were the most effective against most of the tested bacterial strains (Table 2.5) as well as all the tested fungal strains (Table 2.6), with *A. ferox* having MIC values of 0.31 mg/mL against all strains of bacteria except *Micrococcus* species, *A. brasiliensis* and *E. coli*. Plant extracts with MIC values ranging from 0.31 mg/ml to 0.63 mg/ml for antimicrobial activity included *Z. aethiopica* leaves, *B. frutescens* leaves, *E. autumnalis* bulbs and roots, *T. riparia* leaves, *H. limifolia* leaves, *B. natalensis* leaves and roots, *M. plumbea* roots, leaf extracts of *A. aristata*, *A. ferox* and *A. arborescens*, and *H. aethiopicum* leaves. The MIC's values were also successful in highlighting those minimum concentrations at which medicinal plant extracts were most effective. The following crude plant extracts yielded effective minimum concentrations between 0.31 to 0.63 mg/ml for antibacterial activity; the *Aloe* species, *Bulbine* species, *Tetradenia*, *Hypericum*, *Eucomis* and *Zantedeschia* and *Merwillia* species. The antifungal minimum concentrations were good for the above-mentioned plant extracts also.

The broad range activity of the plant extracts against the skin conditions associated with microorganisms suggests that these plant species may offer a degree of efficacy in treating skin conditions like ringworm, ulcers, boils, sunburn, blisters, venereal sores, eczema and inflammation. Traditionally, apart from treating wounds and various skin conditions, the two *Aloe* species had been reported to be used to treat a number of other ailments such as venereal sores, bruises, pimples, blisters, ringworm, boils, gastrointestinal ailments, asthma, constipation, oesophageal cancer, tuberculosis, colds and fever. These species are also used for treating HIV/AIDS opportunistic infections, stomach ache and in childbirth (**HUTCHINGS et al., 1996**;

**CROUCH et al., 2006; DIEDERICHS et al., 2009; KLOS et al., 2009; VAN WYK et al., 2009**). The *Aloe* species contain many interesting secondary metabolites belonging to different classes of compounds and these may be contributing to their healing properties. These species contain many types of compounds, such as alkaloids, anthraquinones, pre-anthraquinones, anthrones, bianthraquinoids, chromones, flavonoids, coumarins and pyrenes. The numerous bioactive compounds isolated from the *Aloe* species include among others, aloe-emodin, conicine, aloesaponol I-IV, aloin A/B, (barbaloin), aloinoside A/B, feroxin A/B, aloesone, lupeol (**DAGNE et al., 2000; GIRELLI et al., 2001**). In addition, other extracts demonstrated good activity on at least two of the tested microorganisms. Extracts, in addition to chloroform for the three *Aloe* species showed significant activity for most of the tested microorganisms; hexane and chloroform extracts of *H. limifolia* and the DCM extract of *H. aethiopicum* (Table 2.5) exhibited good activity against all the fungal strains tested. The number of efficacious drugs available clinically for the treatment of fungal infections is very limited and antimicrobial resistance is one of the major challenges in the treatment of fungal infections (**RUHNKE et al., 1994**). Against this background, significant bioactivity displayed by these plant extracts provide new alternatives for the treatment of fungal infections and thus an opportunity to circumvent the often poorly efficient but expensive fungal treatments.

The Gram-positive bacterium *S. epidermidis* is one of the common causes of skin infections such as pimples, boils, carbuncles, and abscesses (**SINGER and TALAN, 2014**). Resistant strains such as *S. aureus* have become a global challenge and *S. epidermidis* is likely to be added to the list. *H. aethiopicum* (*Un sukumbili*) is reported in a survey conducted on plants among communities in the Ungoye Forest in Northern KwaZulu-Natal, South Africa as the most used plant of choice for healing sores and wounds (**MTHETHWA and DE WET, 2011**). The effective activity shown by this species together with the *Aloe* species against such pathogenic strains as *K. pneumoniae*, *S. epidermidis*, *S. aureus* and all four tested fungi may provide significant information on their use against the skin diseases caused by these pathogens. A study on the stem bark of *Drypetes klainei*, a medicinal plant used for skin conditions and injuries, yielded good wound healing properties and for treatment of burns (**BRUSOTTI et al., 2015**). In a comprehensive review of African medicinal plants, **AGYARE et al. (2016)** noted that more than 61 plants belonging to 36 families have been evaluated for wound healing properties in varying models. Characterisation and isolation of the bioactive compounds remains one of the main areas to be explored in order to exploit the full

benefits of the plant extracts. *In vitro* screening for antibacterial activity of various extracts of plant samples of *T. riparia* was reported to have no inhibitory effects against *B. subtilis*, *E. coli*, *S. aureus* and *K. pneumoniae* (McGAW et al., 2000). However, this study showed good activity against a number of bacteria and fungi with MIC ranges of between 0.63 mg/ml and 0.31 mg/ml. *Hypericum aethiopicum* was reported to be the most used plant for treating wounds and sores in KwaZulu-Natal (MTHETHWA and DE WET, 2011). The differences in results between the agar well diffusion and the disc diffusion assay results may be explained by the rate of growth of bacteria, solvents used to extract the plant compounds, the diverse susceptibility of the bacteria to the plant extracts and the rate of plant extract diffusion.

**Table 2.3** Antibacterial activity (disc diffusion in mm) of the plant extracts examined

Plant	Extract														
		<i>Se(+)</i>	<i>Ec(-)</i>	<i>M(+)</i>	<i>Ea(-)</i>	<i>Kpn(-)</i>	<i>Spn(+)</i>	<i>Ab(+)</i>	<i>Sa(+)</i>	<i>Spy(+)</i>	<i>Bs(+)</i>	<i>Ss(-)</i>	<i>Pae(-)</i>	<i>Pv(-)</i>	<i>Pm(-)</i>
<i>Zantedeschia aethiopica</i> Leaves	Hexane	8	8	8	<b>10</b>	8	0	0	8	0	0	8	0	0	0
	Chloroform	0	8	8	<b>10</b>	<b>10</b>	0	0	8	0	0	8	0	0	0
	DCM	8	8	8	<b>10</b>	0	0	0	8	0	0	<b>10</b>	0	0	0
	Acetone	0	8	8	<b>10</b>	<b>0</b>	0	0	8	0	0	10	0	0	0
	Methanol	0	8	8	<b>10</b>	<b>10</b>	0	0	8	0	0	<b>10</b>	0	0	0
<i>Zantedeschia aethiopica</i> Stems	Hexane	0	8	<b>12</b>	8	<b>10</b>	8	0	0	0	8	<b>10</b>	8	0	<b>10</b>
	Chloroform	0	8	8	8	<b>10</b>	8	<b>10</b>	0	0	8	<b>10</b>	8	0	<b>12</b>
	DCM	0	8	8	8	8	8	<b>10</b>	0	0	8	8	12	0	<b>12</b>
	Acetone	0	8	8	8	<b>10</b>	8	0	0	0	8	8	8	0	<b>12</b>
	Methanol	0	8	<b>10</b>	8	<b>10</b>	8	<b>10</b>	0	0	8	8	8	0	<b>12</b>
<i>Bulbine frutescens</i> Leaves	Hexane	<b>10</b>	0	<b>10</b>	8	0	0	0	<b>10</b>	0	0	8	0	8	8
	Chloroform	8	0	<b>10</b>	8	<b>10</b>	0	0	<b>10</b>	<b>10</b>	0	<b>12</b>	0	<b>10</b>	<b>12</b>
	DCM	<b>10</b>	0	<b>10</b>	8	0	0	0	<b>10</b>	8	0	8	0	8	8
	Acetone	<b>12</b>	0	<b>10</b>	8	0	0	0	8	0	8	8	0	8	8
	Methanol	<b>10</b>	0	<b>10</b>	8	<b>15</b>	0	0	8	0	8	8	0	8	8
<i>Bulbine frutescens</i> Roots	Hexane	8	0	8	8	0	0	0	0	0	8	8	0	<b>12</b>	8
	Chloroform	8	0	8	8	0	0	0	0	0	8	<b>10</b>	0	<b>12</b>	8
	DCM	8	0	<b>14</b>	8	0	0	0	0	0	8	8	0	<b>10</b>	8

	Acetone	8	0	8	8	0	0	0	0	0	8	<b>10</b>	0	<b>10</b>	8
	Methanol	8	0	<b>10</b>	8	0	0	0	0	0	8	8	0	<b>10</b>	8
<i>Eucomis autumnalis</i>	Hexane	8	8	<b>10</b>	<b>10</b>	8	8	8	0	8	8	<b>10</b>	0	<b>10</b>	8
Leaves	Chloroform	8	0	8	8	<b>10</b>	8	8	0	8	8	8	0	<b>10</b>	8
	DCM	8	0	8	<b>12</b>	8	8	8	0	8	8	8	0	<b>10</b>	8
	Acetone	8	0	<b>10</b>	8	8	8	8	0	8	8	8	0	0	8
	Methanol	8	0	<b>10</b>	<b>10</b>	<b>10</b>	8	8	0	8	8	<b>10</b>	0	<b>10</b>	8
<i>Eucomis autumnalis</i>	Hexane	8	0	8	8	0	8	0	8	8	0	0	0	8	0
Bulbs	Chloroform	8	0	8	8	0	8	0	8	8	0	0	0	8	0
	DCM	8	0	8	8	0	8	0	8	8	0	0	0	8	0
	Acetone	8	0	<b>12</b>	8	0	<b>10</b>	0	8	8	0	0	0	8	0
	Methanol	8	0	8	8	0	8	0	8	8	0	0	0	8	0
<i>Eucomis autumnalis</i>	Hexane	8	<b>10</b>	8	<b>10</b>	<b>10</b>	<b>10</b>	0	0	8	<b>12</b>	8	<b>10</b>	8	8
Roots	Chloroform	8	<b>10</b>	8	<b>10</b>	<b>10</b>	<b>10</b>	0	0	<b>10</b>	<b>10</b>	8	8	<b>10</b>	8
	DCM	8	<b>10</b>	8	<b>10</b>	8	8	0	0	8	<b>10</b>	8	<b>10</b>	8	8
	Acetone	8	<b>10</b>	8	<b>10</b>	8	8	0	0	8	<b>10</b>	8	<b>10</b>	<b>10</b>	8
	Methanol	8	8	8	<b>10</b>	<b>10</b>	8	0	0	8	<b>12</b>	8	8	8	8
<i>Tetradenia riparia</i>	Hexane	8	0	8	8	8	0	0	8	8	0	8	0	0	0
Stems	Chloroform	8	0	8	8	8	0	0	8	8	0	8	0	0	0
	DCM	8	0	8	8	8	0	0	0	8	0	8	0	0	0
	Acetone	8	0	8	8	8	0	0	0	8	0	8	0	0	0
	Methanol	8	0	8	8	8	0	0	0	8	0	8	0	0	0

<i>Tetradenia riparia</i>	Hexane	8	0	<b>16</b>	<b>10</b>	0	8	0	0	<b>10</b>	8	8	<b>10</b>	<b>10</b>	0
Leaves	Chloroform	8	0	<b>12</b>	<b>10</b>	0	<b>10</b>	0	0	<b>10</b>	8	8	<b>10</b>	8	0
	DCM	8	0	<b>12</b>	<b>10</b>	0	8	0	0	8	8	8	8	<b>12</b>	0
	Acetone	8	0	<b>10</b>	<b>10</b>	0	<b>10</b>	0	0	8	8	8	8	<b>10</b>	0
	Methanol	8	0	<b>10</b>	<b>10</b>	0	<b>10</b>	0	0	8	8	8	<b>10</b>	8	0
<i>Hypericum aethiopicum</i>	Hexane	8	8	<b>10</b>	8	0	8	8	0	<b>10</b>	8	0	<b>12</b>	8	8
Leaves	Chloroform	<b>10</b>	8	<b>10</b>	8	0	8	8	<b>16</b>	<b>10</b>	8	0	<b>12</b>	<b>10</b>	8
	DCM	8	8	<b>10</b>	8	0	8	8	8	<b>10</b>	8	0	<b>12</b>	<b>10</b>	8
	Acetone	<b>10</b>	8	<b>10</b>	8	0	8	8	<b>10</b>	<b>10</b>	8	0	8	<b>12</b>	8
	Methanol	8	8	<b>10</b>	8	0	<b>10</b>	8	8	<b>10</b>	8	0	8	<b>10</b>	8
<i>Merwillia plumbea</i>	Hexane	8	8	0	8	8	0	8	0	8	0	0	<b>0</b>	0	0
Leaves	Chloroform	8	0	0	8	0	0	8	0	8	0	0	0	0	8
	DCM	8	0	0	8	0	0	8	0	<b>10</b>	0	0	0	0	8
	Acetone	8	0	0	8	0	0	8	0	<b>10</b>	0	0	0	0	8
	Methanol	8	0	0	8	0	0	8	0	<b>10</b>	0	0	0	0	8
<i>Merwillia plumbea</i>	Hexane	<b>10</b>	0	0	<b>10</b>	8	8	8	0	8	0	0	0	0	8
Roots	Chloroform	<b>10</b>	0	0	<b>10</b>	8	8	8	<b>12</b>	8	0	0	<b>10</b>	8	8
	DCM	8	0	0	<b>10</b>	8	<b>10</b>	8	8	8	0	0	8	<b>10</b>	8
	Acetone	<b>10</b>	0	0	<b>14</b>	8	<b>10</b>	8	<b>12</b>	8	0	0	8	8	8
	Methanol	8	0	0	<b>14</b>	8	8	8	8	<b>10</b>	0	0	8	8	8
<i>Merwillia plumbea</i>	Hexane	8	8	0	8	8	0	8	<b>10</b>	0	0	0	8	0	0
Bulbs	Chloroform	8	<b>10</b>	0	<b>10</b>	8	0	8	<b>10</b>	0	0	0	<b>10</b>	0	0



	DCM	8	8	0	8	0	0	8	8	0	0	0	<b>10</b>	0	0
	Acetone	8	<b>10</b>	0	8	0	0	8	8	0	0	0	8	0	0
	Methanol	8	8	0	<b>10</b>	0	0	8	0	0	0	0	8	0	0
<hr/>															
<i>Bulbine natalensis</i>	Hexane	8	0	8	8	0	8	8	8	8	0	0	8	0	8
Leaves	Chloroform	8	0	8	8	0	8	8	8	8	0	0	8	0	8
	DCM	<b>10</b>	0	8	8	0	8	8	<b>10</b>	8	0	0	8	0	8
	Acetone	<b>10</b>	0	8	8	0	8	8	<b>10</b>	8	0	0	8	0	8
	Methanol	<b>10</b>	0	8	8	0	8	8	<b>10</b>	8	0	0	8	0	8
<hr/>															
<i>Bulbine natalensis</i> Roots	Hexane	<b>12</b>	0	0	8	8	8	8	8	<b>10</b>	<b>10</b>	8	0	8	<b>10</b>
	Chloroform	<b>12</b>	0	0	8	8	8	8	8	8	<b>10</b>	8	0	8	<b>10</b>
	DCM	8	0	0	8	8	<b>10</b>	8	<b>10</b>	8	0	8	0	8	8
	Acetone	<b>12</b>	0	0	8	8	<b>12</b>	8	<b>10</b>	8	0	8	0	8	8
	Methanol	<b>12</b>	0	0	8	8	<b>12</b>	8	8	8	0	8	0	8	8
<hr/>															
<i>Aloe aristata</i>	Hexane	8	0	0	8	0	8	0	8	<b>10</b>	0	0	0	0	8
Leaves	Chloroform	8	0	0	8	0	8	0	8	8	0	0	0	0	8
	DCM	8	0	0	8	0	8	0	8	8	0	0	0	0	8
	Acetone	8	0	0	8	0	8	0	8	8	0	0	0	0	8
	Methanol	8	0	0	8	0	8	0	8	<b>10</b>	0	0	0	0	8
<hr/>															
<i>Haworthia limifolia</i>	Hexane	8	8	8	8	8	0	0	<b>10</b>	0	8	0	0	<b>10</b>	0
Leaves	Chloroform	8	8	8	8	8	0	0	<b>10</b>	0	<b>10</b>	0	0	8	0
	DCM	8	8	8	<b>10</b>	8	0	0	<b>10</b>	0	8	0	0	<b>10</b>	0
	Acetone	8	8	8	<b>10</b>	8	0	0	<b>10</b>	0	8	0	0	8	0

	Methanol	8	8	8	<b>10</b>	8	0	0	<b>10</b>	0	8	0	0	<b>10</b>	0
<i>Aloe ferox</i>	Hexane	8	0	0	8	<b>10</b>	8	<b>10</b>	8	<b>10</b>	8	8	9	8	0
Leaves	Chloroform	8	0	0	<b>10</b>	<b>10</b>	<b>10</b>	8	8	<b>10</b>	8	8	9	<b>10</b>	0
	DCM	8	0	0	<b>10</b>	8	8	8	8	8	8	8	9	8	0
	Acetone	8	0	0	8	8	8	<b>10</b>	8	8	8	8	9	<b>10</b>	0
	Methanol	8	0	0	8	8	8	<b>8</b>	10	8	8	8	9	<b>10</b>	0
<i>Aloe arborescens</i>	Hexane	8	0	0	8	8	8	0	<b>10</b>	8	8	0	8	8	8
Leaves	Chloroform	8	8	0	<b>10</b>	<b>10</b>	8	0	<b>10</b>	8	8	0	<b>10</b>	8	8
	DCM	8	0	0	8	<b>10</b>	<b>10</b>	0	<b>10</b>	8	8	0	<b>10</b>	<b>10</b>	8
	Acetone	8	8	0	<b>10</b>	0	<b>10</b>	0	<b>10</b>	8	8	0	0	<b>10</b>	8
	Methanol	8	8	0	8	8	8	0	<b>10</b>	8	<b>18</b>	0	8	8	8
DMSO		0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neomycin		11	10	12	11	11	10	10	10	11	10	12	11	11	10

\*Bacterial species: *Actinomyces brasiliensis* (Ab), *Bacillus spizizeni* (Bs), *Enterobacter aerogenes* (Ea), *Escherichia coli* (Ec), *Klebsiella pneumoniae* (Kp), *Mycobacterium spp* (M), *Proteus mirabilis* (Pm), *Proteus vulgaris* (Pv), *Pseudomonas aeruginosa* (Pa), *Shigella sonnei* (Ss), *Staphylococcus aureus* (Sa), *Staphylococcus epidermidis* (Se), *Streptococcus pneumoniae* (Spn), *Streptococcus pyogenes* (Spy).

(+) Gram-positive; (-) Gram-negative

**Table 2.4** Antifungal activity (Disc Diffusion in mm) of the plant extracts examined

Antifungal Activity		*Fungal species (inhibition zones averages mm)			
Plant	Extract	<i>Tr</i>	<i>Tm</i>	<i>Ca</i>	<i>Ct</i>
<i>Zantedeschia</i>	Hexane	<b>10</b>	0	8	8
<i>aethiopica</i>	Chloroform	8	8	8	8
Leaves	DCM	8	8	8	<b>10</b>
	Acetone	<b>10</b>	8	8	<b>10</b>
	Methanol	8	8	<b>10</b>	<b>10</b>
<i>Zantedeschia</i>	Hexane	8	<b>10</b>	8	0
<i>aethiopica</i>	Chloroform	8	<b>10</b>	8	0
Stems	DCM	8	8	8	8
	Acetone	8	8	8	8
	Methanol	8	8	8	8
<i>Bulbine</i>	Hexane	<b>10</b>	8	8	8
<i>frutescens</i>	Chloroform	<b>10</b>	8	<b>10</b>	8
Leaves	DCM	<b>10</b>	8	<b>10</b>	8
	Acetone	<b>10</b>	<b>10</b>	<b>10</b>	8
	Methanol	<b>10</b>	<b>10</b>	<b>10</b>	8
<i>Bulbine</i>	Hexane	0	9	8	8
<i>frutescens</i>	Chloroform	0	8	8	<b>10</b>
Roots	DCM	0	0	8	8
	Acetone	0	0	8	<b>12</b>
	Methanol	0	0	8	8
<i>Eucomis</i>	Hexane	8	8	8	<b>10</b>
<i>autumnalis</i>	Chloroform	8	8	8	8

Leaves	DCM	8	8	8	8
	Acetone	8	8	<b>10</b>	8
	Methanol	8	<b>10</b>	<b>10</b>	<b>10</b>
<i>Eucomis autumnalis</i>	Hexane	<b>10</b>	8	8	<b>10</b>
	Chloroform	<b>10</b>	8	8	<b>10</b>
Bulbs	Acetone	8	8	8	8
	DCM	8	8	8	8
	Methanol	8	8	8	<b>10</b>
<i>Eucomis autumnalis</i>	Hexane	0	8	<b>10</b>	8
	Chloroform	0	8	<b>10</b>	0
Roots	DCM	0	8	<b>10</b>	8
	Acetone	0	8	<b>10</b>	0
	Methanol	0	8	<b>10</b>	0
<i>Tetradenia riparia</i>	Hexane	<b>10</b>	8	8	8
	Chloroform	<b>10</b>	8	0	0
Stems	DCM	8	8	0	0
	Acetone	<b>10</b>	<b>10</b>	8	0
	Methanol	<b>10</b>	<b>10</b>	<b>10</b>	8
<i>Tetradenia riparia</i>	Hexane	<b>10</b>	8	8	<b>12</b>
	Chloroform	8	8	8	8
Leaves	DCM	<b>10</b>	8	8	<b>12</b>
	Acetone	<b>10</b>	<b>10</b>	8	<b>10</b>
	Methanol	<b>10</b>	<b>10</b>	8	8
<i>Haworthia limifolia</i>	Hexane	0	8	<b>10</b>	<b>10</b>
	Chloroform	<b>16</b>	8	0	0
Leaves	DCM	8	8	<b>14</b>	0
	Methanol	8	10	8	0

	Acetone	<b>10</b>	<b>12</b>	8	0
<i>Merwillia plumbea</i> Leaves	Hexane	<b>10</b>	0	8	8
	Chloroform	<b>10</b>	0	<b>12</b>	<b>14</b>
	DCM	8	8	8	8
	Acetone	8	<b>10</b>	8	8
	Methanol	0	0	8	8
<i>Bulbine natalensis</i> Roots	Hexane	8	8	<b>10</b>	8
	Chloroform	8	8	<b>12</b>	8
	DCM	<b>10</b>	8	<b>10</b>	<b>10</b>
	Acetone	<b>10</b>	8	8	0
	Methanol	8	8	<b>10</b>	0
<i>Merwillia plumbea</i> Leaves	Hexane	0	8	8	0
	Chloroform	0	8	<b>10</b>	<b>10</b>
	DCM	0	<b>10</b>	8	0
	Acetone	0	8	10	8
	Methanol	0	8	8	0
<i>Merwillia plumbea</i> Roots	Hexane	<b>12</b>	<b>10</b>	<b>10</b>	8
	Chloroform	<b>12</b>	<b>12</b>	<b>12</b>	8
	DCM	8	8	8	8
	Acetone	<b>12</b>	<b>10</b>	<b>10</b>	8
	Methanol	8	8	8	8
<i>Bulbine natalensis</i> Leaves	Hexane	8	8	<b>16</b>	<b>12</b>
	Chloroform	8	8	<b>10</b>	8
	DCM	<b>10</b>	<b>10</b>	<b>10</b>	<b>12</b>
	Acetone	<b>10</b>	8	<b>14</b>	8
	Methanol	<b>10</b>	8	<b>10</b>	8
<i>Aloe aristata</i>	Hexane	8	<b>10</b>	8	8

Leaves	Chloroform	8	<b>10</b>	8	8
	DCM	8	8	<b>10</b>	8
	Acetone	8	<b>10</b>	8	8
	Methanol	8	<b>10</b>	8	<b>10</b>
<i>Haworthia</i>	Hexane	<b>10</b>	8	<b>12</b>	<b>12</b>
<i>limifolia</i>	Chloroform	<b>10</b>	<b>10</b>	8	8
Leaves	DCM	<b>10</b>	8	<b>10</b>	<b>10</b>
	Acetone	<b>10</b>	8	<b>10</b>	<b>10</b>
	Methanol	<b>10</b>	<b>10</b>	8	<b>10</b>
<i>Aloe ferox</i>	Hexane	8	0	8	8
Leaves	Chloroform	8	0	<b>10</b>	<b>10</b>
	DCM	8	8	<b>10</b>	<b>10</b>
	Acetone	8	8	<b>10</b>	8
	Methanol	<b>10</b>	8	10	8
<i>Aloe arborescens</i>	Hexane	<b>10</b>	0	8	0
	Chloroform	8	8	<b>10</b>	<b>10</b>
Leaves	DCM	8	0	8	8
	Acetone	8	8	<b>10</b>	8
	Methanol	<b>10</b>	8	8	0
DMSO		0	0	0	0
Amphotericin B		11	10	12	11

\*Fungal strains: *Trichophyton rubrum* (Tr), *Trichophyton mentagrophytes* (Tm), *Candida albicans* (Ca), *Candida tropicalis* (Ct).

**Table 2.5** Antibacterial activity (MIC mg/mL) of the plant extracts examined

<b>*Bacterial strains</b>																
<b>Gram-positive</b>										<b>Gram-negative</b>						
<b>Plant</b>	<b>Extract</b>	<b>Part</b>	<b>M</b>	<b>Bs</b>	<b>Spn</b>	<b>Sa</b>	<b>Se</b>	<b>Ab</b>	<b>Spy</b>	<b>Ea</b>	<b>Kp</b>	<b>Pv</b>	<b>Pa</b>	<b>Pm</b>	<b>Ss</b>	<b>Ec</b>
<i>Zantedeschia aethiopica</i>	Chloroform	Leaves	2.50	<b>0.63</b>	2.50	2.50	2.50	2.50	2.50	2.50	1.25	2.50	2.50	<b>0.31</b>	2.50	2.50
	Chloroform	Stems	1.25	2.50	2.50	2.50	2.50	2.50	<b>2.50</b>	1.25	1.25	2.50	2.50	<b>0.63</b>	2.50	2.50
	Methanol	Leaves	1.25	<b>0.63</b>	2.50	2.50	2.50	2.50	2.50	1.25	1.25	2.50	2.50	<b>0.63</b>	2.50	2.50
<i>Bulbine frutescens</i>	Chloroform	Leaves	1.25	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	2.50	2.50	<b>0.63</b>	1.25	1.25	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	2.50	2.50
	Chloroform	Bulbs	1.25	2.50	1.25	2.50	<b>0.63</b>	<b>0.63</b>	2.50	1.25	1.25	2.50	2.50	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
	Methanol	Leaves	1.25	2.50	2.50	<b>0.63</b>	2.50	2.50	2.50	1.25	1.25	<b>0.63</b>	2.50	2.50	2.50	2.50
<i>Eucomis autumnalis</i>	Chloroform	Bulbs	1.25	2.50	1.25	2.50	2.50	<b>0.63</b>	2.50	1.25	1.25	2.50	2.50	<b>0.63</b>	2.50	2.50
	Chloroform	Roots	1.25	<b>0.31</b>	1.25	2.50	2.50	2.50	<b>0.63</b>	1.25	1.25	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	2.50	2.50
	DCM	Leaves	1.25	2.50	1.25	2.50	2.50	<b>0.63</b>	2.50	1.25	1.25	2.50	2.50	<b>0.63</b>	2.50	2.50
	Acetone	Leaves	1.25	2.50	1.25	2.50	<b>0.63</b>	<b>0.63</b>	2.50	1.25	1.25	2.50	2.50	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
	Acetone	Bulbs	1.25	2.50	1.25	2.50	2.50	<b>0.63</b>	2.50	1.25	1.25	2.50	2.50	<b>0.63</b>	2.50	2.50
	Acetone	Roots	1.25	<b>0.63</b>	1.25	<b>0.63</b>	2.50	2.50	<b>0.63</b>	1.25	1.25	<b>0.63</b>	<b>0.63</b>	2.50	2.50	2.50
<i>Tetradenia riparia</i>	Hexane	Leaves	1.25	<b>0.31</b>	1.25	2.50	2.50	1.25	<b>0.63</b>	1.25	2.50	<b>0.63</b>	<b>0.63</b>	2.50	1.25	<b>0.63</b>
	Chloroform	Leaves	2.50	<b>0.63</b>	1.25	<b>0.63</b>	2.50	1.25	<b>0.63</b>	2.50	<b>0.63</b>	2.50	2.50	1.25	1.25	2.50
	Chloroform	Stems	1.25	2.50	1.25	2.50	2.50	<b>0.63</b>	2.50	1.25	1.25	2.50	2.50	2.50	2.50	2.50
	Acetone	Stems	1.25	2.50	1.25	2.50	2.50	<b>0.63</b>	2.50	1.25	2.50	2.50	2.50	2.50	2.50	2.50
	Acetone	Leaves	<b>0.63</b>	<b>0.63</b>	1.25	<b>0.63</b>	1.25	1.25	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	2.50	2.50	1.25	1.25	2.50
	Methanol	Leaves	2.50	2.50	1.25	<b>0.63</b>	1.25	1.25	2.50	<b>0.63</b>	2.50	2.50	<b>0.63</b>	1.25	1.25	2.50

<i>Haworthia aethiopicum</i>	Chloroform	Leaves	<b>0.31</b>	<b>0.31</b>	<b>0.63</b>	<b>0.31</b>	1.25	1.25	<b>0.31</b>	<b>0.63</b>	<b>0.31</b>	2.50	2.50	1.25	1.25	2.50
	DCM	Leaves	<b>0.63</b>	<b>0.63</b>	2.50	<b>0.63</b>	1.25	1.25	<b>0.31</b>	<b>0.63</b>	<b>0.63</b>	2.50	2.50	1.25	1.25	2.50
	Acetone	Leaves	<b>0.63</b>	<b>0.63</b>	1.25	<b>0.63</b>	1.25	1.25	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	2.50	2.50	1.25	1.25	2.50
<i>Merwillia plumbea</i>	Hexane	Leaves	<b>0.63</b>	<b>0.63</b>	1.25	<b>0.63</b>	1.25	1.25	<b>0.16</b>	2.50	2.50	<b>0.63</b>	<b>0.63</b>	1.25	1.25	2.50
	Chloroform	Bulbs	1.25	2.50	1.25	<b>0.63</b>	1.25	1.25	2.50	1.25	1.25	1.25	2.50	<b>0.63</b>	1.25	2.50
	DCM	Leaves	2.50	2.50	1.25	<b>0.63</b>	1.25	1.25	2.50	1.25	1.25	1.25	2.50	<b>0.63</b>	1.25	2.50
	Acetone	Leaves	1.25	2.50	1.25	2.50	2.50	2.50	2.50	1.25	1.25	1.25	2.50	<b>0.63</b>	1.25	2.50
	Methanol	Bulbs	2.50	2.50	1.25	2.50	1.25	1.25	<b>0.63</b>	1.25	1.25	1.25	2.50	1.25	1.25	2.50
<i>Bulbine natalensis</i>	Hexane	Leaves	2.50	<b>0.63</b>	1.25	<b>0.63</b>	2.50	2.50	<b>0.63</b>	1.25	1.25	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	1.25	2.50
	Chloroform	Bulbs	1.25	<b>0.63</b>	1.25	<b>0.63</b>	<b>0.63</b>	2.50	<b>0.63</b>	1.25	2.50	1.25	2.50	<b>0.63</b>	1.25	2.50
<i>Aloe aristata</i>	Hexane	Leaves	2.50	2.50	1.25	<b>0.63</b>	<b>0.63</b>	2.50	1.25	1.25	2.50	1.25	2.50	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
	DCM	Leaves	1.25	2.50	1.25	<b>0.63</b>	<b>0.63</b>	2.50	2.50	1.25	2.50	2.50	2.50	<b>0.63</b>	1.25	2.50
	Acetone	Leaves	1.25	2.50	2.50	<b>0.63</b>	<b>0.63</b>	2.50	2.50	1.25	2.50	2.50	2.50	<b>0.16</b>	1.25	2.50
<i>Haworthia limifolia</i>	Hexane	Leaves	2.50	2.50	2.50	<b>0.63</b>	<b>0.63</b>	2.50	<b>0.63</b>	2.50	2.50	2.50	2.50	<b>0.63</b>	2.50	2.50
	Chloroform	Leaves	1.25	1.25	1.25	<b>0.63</b>	<b>0.63</b>	1.25	<b>0.63</b>	1.25	1.25	1.25	1.25	<b>0.63</b>	1.25	2.50
<i>Aloe ferox</i>	Chloroform	Leaves	<b>0.63</b>	<b>0.31</b>	<b>0.31</b>	<b>0.31</b>	<b>0.31</b>	<b>0.63</b>	<b>0.31</b>	<b>0.31</b>	<b>0.31</b>	<b>0.31</b>	<b>0.31</b>	<b>0.31</b>	<b>0.31</b>	<b>0.63</b>
<i>Aloe arborescens</i>	DCM	Leaves	1.25	<b>0.31</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.31</b>	<b>0.63</b>	1.25	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
Neomycin µg/ml			0.16	0.16	0.16	0.16	0.16	0.08	0.16	0.08	0.16	0.16	0.16	0.16	0.16	0.16

\*Bacterial strains: *Actinomyces brasiliensis* (Ab), *Bacillus subtilis* (Bs), *Micrococcus species* (M), *Staphylococcus aureus* (Sa), *Staphylococcus epidermidis* (Se), *Streptococcus pneumoniae* (Sp), *Streptococcus pyogenes* (Spy), *Enterobacter aerogenes* (Ea), *Klebsiella pneumonia* (Kpn), *Pseudomonas aeruginosa* (Pa), *Shigella sonnei* (Ss), *Proteus mirabilis* (Pm), *Proteus vulgaris* (Pv), *Escherichia coli* (Ec). Values written in bold are considered active (< 1 mg/mL).



**Table 2.6** Antifungal activity (MIC mg/mL) of the plant extracts examined

Plant	Extract	Part	*Fungal strains			
			Tr	Tm	Ca	Ct
<i>Zantedeschia aethiopica</i>	Chloroform	Stems	<b>0.63</b>	<b>0.63</b>	1.25	1.25
	Acetone	Stems	1.25	<b>0.63</b>	1.25	1.25
	Methanol	Leaves	<b>0.63</b>	1.25	1.25	<b>0.63</b>
<i>Eucomis autumnalis</i>	Chloroform	Bulbs	1.25	1.25	1.25	<b>0.63</b>
	Acetone	Leaves	1.25	1.25	1.25	<b>0.63</b>
<i>Tetradenia riparia</i>	Chloroform	Stems	<b>0.31</b>	<b>0.63</b>	1.25	1.25
	Acetone	Stems	1.25	<b>0.63</b>	<b>0.63</b>	1.25
	Methanol	Leaves	<b>0.63</b>	<b>0.63</b>	1.25	1.25
<i>Merwillia plumbea</i>	Hexane	Leaves	1.25	<b>0.63</b>	1.25	<b>0.63</b>
	Chloroform	Leaves	<b>0.63</b>	<b>0.63</b>	1.25	<b>0.63</b>
	Chloroform	Bulbs	<b>0.63</b>	<b>0.63</b>	<b>0.31</b>	1.25
	Acetone	Bulbs	1.25	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
	Acetone	Roots	1.25	<b>0.63</b>	1.25	<b>0.63</b>
	Methanol	Roots	1.25	<b>0.63</b>	1.25	<b>0.63</b>
<i>Bulbine natalensis</i>	Chloroform	Leaves	1.25	<b>0.31</b>	<b>0.63</b>	<b>0.63</b>
<i>Bulbine frutescens</i>	Chloroform	Leaves	1.25	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
<i>Aloe aristata</i>	Hexane	Leaves	<b>0.31</b>	1.25	<b>0.63</b>	<b>0.63</b>
	DCM	Leaves	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
	Acetone	Leaves	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
<i>Haworthia limifolia</i>	Hexane	Leaves	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
	Chloroform	Leaves	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
<i>Aloe ferox</i>	Chloroform	Leaves	<b>0.63</b>	<b>0.31</b>	<b>0.63</b>	<b>0.63</b>
	DCM	Leaves	<b>0.63</b>	<b>0.31</b>	<b>0.63</b>	<b>0.63</b>
<i>Hypericum aethiopicum</i>	Chloroform	Leaves	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
<i>Aloe arborescens</i>	DCM	Leaves	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>	<b>0.63</b>
Amphotericin B (µg/ml)			0.93	0.93	0.93	0.93

\*Fungal strains: *Candida albicans* (Ca), *Candida tropicalis* (Ct), *Trichophyton rubrum* (Tr), *Trichophyton mentagrophytes* (Tm). Values written in bold are considered active (< 1 mg/mL)

## 2.8 Conclusions

The study indicated the antimicrobial activity of the eleven medicinal plant extracts commonly identified by traditional doctors having a significant effect on wound healing and dermatological disorders. Various plant parts were screened in the disc diffusion and the minimum inhibitory concentration assays. Leaves were shown to have more activity and their activity are compared to the other plant parts like the stems, rhizomes, bulbs, and roots. The assays showed that many of the selected medicinal plants were effective as antibacterial and antifungal agents against a wide range of commonly occurring pathogens causing infection and interfering with effective wound healing and dermatological conditions. The extracts from the leaves of these plants are reported to be effective for wound healing by traditional healers and this may be due to their antimicrobial activity. Through scientific evidence and research it can be proven that all underground parts like roots, rhizomes and the stems could be left to grow and are sustainable promoting the harvesting of the leaves only rather than whole plants of which much is wasted and discarded when harvested for traditional medicinal health care.

The eleven plant species were tested against 14 microorganisms and showed inhibition for most of these at varying levels. Inhibition zones above ten mm were used as a guideline to assess the MIC's and concentration levels range from 0.31 mg/ml to 0.63 mg/ml for the highly active antimicrobial plant extracts. The following plant extracts showed these outcomes: *Z. aethiopica* leaves, *B. frutescens* leaves, *E. autumnalis* bulb and roots, *T. riparia* leaves, *H. limifolia* leaves, *B. natalensis* leaves and roots, *M. plumbea* roots, leaf extracts of *A. aristata*, *A. ferox* and *A. arborescens*, and *H. aethiopicum* leaves. The MIC's were also successful in highlighting minimum concentrations at which medicinal plant extracts were most effective. The following crude plant extracts reported effective minimum concentrations between 0.31 to 0.63 mg/ml for antibacterial activity; the *Aloe* species, *Bulbine* species, *Tetradenia*, *Hypericum*, *Eucomis* and *Zantedeschia* and *Merwillia*. The antifungal minimum concentrations were good for the above-mentioned plant extracts also. Further investigations into antimicrobial screening for medicinal plants is necessary for future public health care as the rising costs of allopathic medicines become unaffordable to the majority of the country's population. It is recommended that further, more intensive antimicrobial screening be conducted to identify bioactive compounds that are effective against multidrug resistant microbial strains that would be significant for clinical management of infectious pathogens.

# CHAPTER THREE

## PHENOLIC CONTENT

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### 3.1 Introduction

The sustainable advancement of infectious diseases and the progress of multi-drug resistance by pathogens to developed pharmaceuticals, have led to the extension of the exploration for new unique leads against parasitic, bacterial, fungal and viral infections (**GIBBONS, 2004**). Even with the recent advances in drug development, natural plant-derived compounds are still proving to be an invaluable source of treatments and management for human's diseases (**SALIM et al., 2008**). Plant-derived antimicrobials have a long history of providing abundant desired novel therapeutics (**AVILA et al., 2008**). Plants continuously interrelate with the rapidly shifting and potentially detrimental external environmental factors. Plants have changed intricate alternative defence strategies that involve a massive variability of chemical metabolites as tools to overcome conditions of stress (**NCUBE et al., 2012a**). The ability of plants to carry out combinatorial chemistry by matching, mixing and evolving the gene products necessary for secondary metabolite biosynthetic pathways, produces an extension pool of chemical compounds, which humans have used to their benefit. The use of plants by humans in both traditional and allopathic medicinal systems, therefore, largely gains phenomenal accomplishments and successes (**NCUBE et al., 2012a**).

A number of traditionally used medicinal plants have to date been screened for various biological activities in both *in vivo* and *in vitro* models. The chemical exploration and purification of plant extracts claimed to have medicinal capacities have produced an abundance of purified compounds proven to be essential in the practice of modern medicine (**GOLDSTEIN et al., 1994; TYLER et al., 1988**). However, in traditional medicine, these compounds are largely used in crude extracts in the form of herbal remedies (**PUJOL, 1990**). Considerations of the rapid development of newer emerging infectious diseases and the expansion of multi-drug resistance where existing curatives occur, one of the strategies employed in indigenous plant medicine is the mixture of herbal components in the remedies. Traditional remedies are often produced by combining several diverse plant species. The pharmacological effects of such mixtures could be as a result of the sum of different classes of compounds with diverse mechanisms of action. Reported studies on the total contents of a

herbal creation reveal a significantly improved effect than an equivalent dose of or a single constituent herb or one isolated active ingredient (**WILLIAMSON, 2001; NAHRSTEDT and BUTTERWECK, 2010**). These findings suggest that, the effects may arise from synergistic mechanisms of herbal ingredients. The relationship of synergism happens when more than two compounds cooperate in ways that mutually amplify, enhance, or potentiate each other's effect more meaningfully than the modest effect of single ingredients (**WILLIAMSON, 2001**).

There is noteworthy information on the bioactivity of screened medicinal plant extracts, however in most studies these findings are reported on the basis of isolated classes or groups of compounds extracted using diverse individual solvents. As indicated earlier many scientists perform extraction using solvents with different polarities, e.g. petroleum ether, ethyl acetate, ethanol chloroform and water (**WILLIAMSON, 2001; NAHRSTEDT and BUTTERWECK, 2010**). The quality and the overall quantity of the plants extracted compounds in any given species would vary largely as a function of the type of solvent used and the growing season. In light of the diversity of the phytochemical compounds formed within an individual plant, an examination into this aspect would possibly reveal the concealed potentialities of the therapeutic and medicinal values of the phytochemical compounds (**WILLIAMSON, 2001; NAHRSTEDT and BUTTERWECK, 2010**). Assessing the ethnopharmacological and ethnobotanical knowledge as a selection strategy gives priority to traditionally based medicinal plants used for skin, wound healing, isolating and identifying bioactive compounds. Pharmacological and phytochemical studies of the investigated eleven medicinal plant species was necessary to validate the usage of the plants in skin disorders and wound healing. Furthermore, this provides sufficient information for the separation and documentation of active compounds that are present in the investigated plants.

In this study in order to understand the source of the plant's pharmacological activities, total compounds of a phenolic nature including condensed tannins and proanthocyanidins were quantitatively investigated in terms of their amounts in extracts of the plant materials using spectrophotometric methods.

### **3.1.1 Phenolic Compounds**

In organic chemistry, phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. The simplest of the class is phenol, which is also called carboic acid  $C_6H_5OH$ . Phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the

molecule. Plant phenolics represent a very large group of defensive compounds defined as having a phenol moiety. The phenolics range in complexity from simple phenolics and quinines, through chalcones and stilbenes to a range of phenolics with three rings namely anthocyanins, anthochlors, benzofurans, chromones, coumarins, flavonoids, isoflavonoids, neoflavanoids, stibenoids and xanthones. More complex polycyclic phenolics exist, notably the hydrolysable tannins and condensed tannins (**WILLIAMSON, 2001; NAHRSTEDT and BUTTERWECK, 2010**). Notable simple phenol-related odorants include 4-methoxybenzaldehyde, guaiacol, 4-hydroxybenzaldehyde, phenylethyl alcohol, piperonal and vanilla. Some simple phenolics inhibit cyclooxygenase (COX) enzymes and 5-lipoxygenase (5-LOX), and have antimicrobial properties, particularly against phytopathogenic organisms (**BRUNETON, 1995; POLYA, 2003**). Phenolic compounds are of important pharmacological value which include antimicrobial and anti-inflammatory activities.

In higher plants, hydrolysable and condensed tannins are the two groups distinguished based on their structure and biogenetic origin (**BRUNETON, 1995**). Hydrolysable tannins are esters of a sugar and of variable number of phenolic acid molecules, while condensed tannins or proanthocyanidins are polymeric flavans. Tannins dissolve in water to form colloidal solutions, but their solubility varies with their degree of polymerisation. Most of the biological properties of tannins are linked to their capacity to form multiplexes with proteins particularly and other macromolecules. Externally, they waterproof the external layers of the skin and the mucosa, thus protecting the underlying layers. They are also known to have anti-diarrheal properties. They also have blood vessel constriction effects on minor superficial vessels and heighten tissue restoration in the case where superficial wounds or burns lose fluid thus reducing fluid loss and inhibiting external antagonisms (**BRUNETON, 1995**).

Plant flavonoids are known as nutraceutical compounds, as precursors for the food that provide medical or health benefits including the prevention and treatment of disease. These are non-toxic food extract supplements scientifically proven for their antioxidant activity, antimicrobial activity and antiviral activity having effects on the gastrointestinal system/antiulcer activity, hepatoprotective activity, anti-inflammatory activity, antidiabetic effects, cardiovascular system/vasorelaxant, antiatherosclerosis, antithrombotic effects, cardio protective effects, antineoplastic activity and on the central nervous system (**TAPAS et al., 2008**).

## **3.2 Materials and Methods**

### **3.2.1 Preparation of Selected Plants for Phenolic Quantification**

Plant material was collected and processed as described in Chapter 2. Compounds that are phenolic in nature were extracted from plant material and as described by **MAKKAR (1999)** with minor adjustments. Plant material that had been dried (2 g) were extracted for 20 min using 20 ml of 50% (v/v) aqueous methanol by sonication on ice. The extracts were then filtered through Whatman No. 1 filter paper under vacuum and the extracted polyphenols were quantified from these filtered extracts.

### **3.2.2 Determination of Phenolic composition**

#### **3.2.2.1 Total Phenolics**

Total phenolic content in plant samples were determined using the Folin-Ciocalteu (Folin C) assay as described by **MAKKAR (1999)** with slight modifications. Fifty microliters of each extract were transferred into test tubes into which 950  $\mu$ l distilled water were added followed by 1 N Folin C reagent (500  $\mu$ l) and 2% sodium carbonate (2.5 ml). The test mixtures were incubated for 40 min at room temperature and the absorbance read at 725 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia) against a blank consisting of aqueous methanol instead of extract. Total phenolic concentrations were expressed in mg gallic acid equivalents (GAE) per g dry weight (DW).

#### **3.2.2.2 Determination of Proanthocyanidins/Condensed Tannins**

The butanol-HCl assay (**PORTER et al, 1985**) was used to quantify proanthocyanidin content. Three millilitres of butanol-HCl reagent (95:5, v/v) were added to 500  $\mu$ l of each extract, followed by 100  $\mu$ l ferric reagent (2% ferric ammonium sulphate in 2 N HCl). The test combination was mixed by Vortex and placed in a boiling water bath for 60 min, absorbance was then read at 550 nm using a UV-vis spectrophotometer against a blank prepared in a similar way without heating. Each sample extract had three replicates. Proanthocyanidins (mg LCE/g DW) were calculated as leucocyanidin equivalents (LCE) using the formula developed by **PORTER et al. (1985)**.

#### **3.2.2.3 Determination of Flavonoids**

The vanillin assay, as described by **HAGERMAN (2002)**, was used to determine flavonoid content from plant samples. Extracts (50  $\mu$ l), were made up to 1 ml with methanol in test tubes

before adding 2.5 ml methanolic-HCl (95:5, v/v) and 2.5 ml vanillin reagent (1 g/100 ml acetic acid). Similar preparations of a blank that contained methanol instead of plant extract were made. After 20 min incubation at room temperature, absorbance was read at 500 nm using a UV-Vis spectrophotometer. A catechin standard curve was prepared from 1 mg ml<sup>-1</sup> catechin (Sigma-Aldrich) stock solution in methanol. The flavonoid content was expressed as µg catechin equivalents (CTE) per g DW. Each sample extract had three replicates.

### 3.3 Results and Discussion

Table 3 presents the total phenolic, flavonoid and proanthocyanidin contents of the eleven medicinal plants screened. Total phenolic levels, flavonoids and tannins were significant for the three *Aloe* species, *Haworthia* and *Hypericum* species. Phytochemical screening of natural products underpins the development of cosmetic and anti-ageing products. Phenolic compounds, particularly tannins and flavonoids are known to enhance rapid skin regeneration and antimicrobial effects. Although the bioactivity of the secondary metabolites, including phenolic compounds, is a function of both quality and quantity as well as interactions of these metabolites, their respective quantities in an extract sometimes gives a correlation with activity. In skin burns and wound healing processes, phenolic-protein complexes form a film that prevents dehydration and creates a physical barrier to damaged tissue thereby preventing microbial infection and chemical damage (LUSEBA et al., 2007).

It is through the same mechanism (phenolic-protein complexing) that these groups of compounds are thought to exert their antimicrobial effects. The concentration of total phenolics in the investigated plant samples ranged from 0.05 to 4.69 mg/g DW. The highest concentrations of total phenolic compounds were found in the leaves of the three *Aloe* species (*A. arborescens*, *A. aristata* and *A. ferox*) followed by *H. limifolia* and *H. aethiopicum*. Coincidentally, the extracts from these five species also demonstrated good antimicrobial activity against a number of tested microorganisms. Although it is insufficient to draw solid conclusions from just one group of compounds tested in this study considering the multitude of compounds produced by plants, the correlation between the trend in the amount of phenolic compounds and antimicrobial effects, it is tempting to speculate on the involvement of these compounds. Phenolic compounds in plants are purported to serve defence roles against invading pathogens (DEY and HARBORNE, 1989; TREUTTER, 2001) and important pharmacological activities such as antioxidants, anticarcinogenic, antibacterial, and anti-

inflammatory effects have been recorded (**SHARMA et al., 1994; BRUNETON, 1995; KUDA et al., 2005**).

A similar trend to that of total phenolics were observed for flavonoids and condensed tannins (proanthocyanidins). Flavonoids are known for direct antibacterial properties, synergistic activity with antibiotics and the ability to suppress bacterial virulence; in addition to having anti-carcinogenic properties (**TAPAS et al., 2008**). Tannins are known for antibacterial, anti-parasitic, anti-inflammatory and anti-ulcer properties. The proanthocyanidin content ranged from 0.09 to 9.85  $\mu\text{g/g}$  DW with the highest content in *H. aethiopicum*, followed by *A. ferox*, *A. aristata*, *H. limifolia*, *B. natalensis* and *A. arborescens* respectively. Tannins are reported to be the key candidates in the phenolic-protein complexes. The hydroxyl groups in phenolic compounds are excellent hydrogen donors that forms strong hydrogen bonds with protein carboxyl groups. Apart from wound healing, the phenolic-protein properties could exert the antimicrobial and enzyme inhibitory properties by forming hydrophobic interactions with the protein regions of the bacterial cell wall (**NCUBE and VAN STADEN, 2015**).

All the plant extracts evaluated showed considerable amounts of phenolic compounds that could in one way or another be attributed to the pharmacological activities observed in Table 3.1. It is however noted that plant extracts with high phenolic contents showed moderate to high antimicrobial and anti-inflammatory activities. This suggest that the value of the compounds of phenolic nature plays a major role in skin disorders and wound healing.

Condensed tannins are measured as leucocyanidin (flavan-3, 4-diol) equivalents which are products of the breakdown of condensed tannins in the assay (**SHARON et al., 2002; CROZIER et al., 2006a; 2006b**). According to **BRUNETON (1995)**, condensed tannins are reported to accumulate in leaf and root tissues of many plants, probably as a defence mechanism against microorganisms. Condensed tannins or proanthocyanidins essentially contain positive gallo catechin and negative epicatechin, positive catechin and epigallocatechin and their derivatives via carbon- carbon links. These compounds are primarily responsible for inhibiting the generation of chemiluminescence by activated human polymorphonuclear neutrophil (PMN) indicating that these compounds inhibit the oxidative outburst of PMN inflammation (**POLYA, 2003**).

Several flavonoids and bioflavonoids are potent inhibitors of lipoxygenase or cyclooxygenase (**BRUNETON, 1995; POLYA, 2003**). According to **TALHOUK et al. (2007)**, the flavonoids



are known to act on the inflammatory response via many routes and block molecules like COX, iNOS, cytokines, nuclear factor-kB and matrix metalloproteinases. Some flavonoids have been described to be active against acute inflammation *in vivo* using a carcinogenic-induced mouse paw oedema model (**PELZER et al., 1998**). Flavonoids could be major bioactive compounds responsible for the anti-inflammatory properties exhibited by plant extracts. Furthermore, flavonoid derivatives are reported to inhibit the development of fluids that result in diarrhoea and abdominal pains (**SCHUIER et al., 2005**).

Polyphenols or phenolic compounds are characterised by at least one aromatic ring bearing one or more hydroxyl substituents and other functional derivatives such as methyl esters, esters and glycosides (**DEY and HARBORNE, 1989**). These are a diverse group of higher secondary metabolites, with derivatives of the shikimate pathway, phenylpropanoid metabolism pentose phosphate and phenylalanine as the pioneer molecules (**RYAN et al., 1999; BALASUNDRAM et al., 2006**). The phenylpropanoid pathway is a very important metabolic pathway in plant metabolism in terms of carbon flux, with more than 20% of the cell total metabolism going through this process (**DIXON and PAIVA, 1995**). Polyphenol pathways yield lignans, lignins flavonoids and anthocyanins. The enzyme PAL transforms phenylalanine into trans-cinnamic acid using a non-oxidative deamination process. The enzyme shows an important part in the pathways fluidity. Basic phenol is the unit of polyphenols and are ubiquitous in plants. The type and compound produced differ amongst plants (**CROZIER et al., 2006a; 2006b**). Phenolic compounds are integral in cell wall structure as polymeric materials like lignins which provide mechanical strength and support forming a barrier against invading microorganisms (**WALLACE and FRY, 1994**). The complexes display a significant free radical scavenging ability which is determined fundamentally by their reactivity as electron- or hydrogen- donating agents and the steadiness of the resulting anti-oxidant derived radicals, which prevents oxidation of several food ingredients, particularly oils and fatty acids (**RICE-EVANS et al., 1997; SUBBA RAO and MURALIKRISHNA, 2002**). Tannins and flavonoids are among the most comprehensive assemblies of phenolic compounds.

Flavonoids are the most well-known and varied class of phenolics with low molecular weight (**HEIM et al, 2002**). They are derived biosynthetically from an arrangement of the acetate and shikimic acid pathways (**WATERMAN and MOLE, 1994**). They are elements of plant parts with innumerable variants (**HEIM et al., 2002; CROZIER et al., 2006a; 2006b**) and have high concentrations in leaf epidermal tissue and fruit peels (**HRAZDINA, 1992; CROZIER et al., 2006a; 2006b**). Minor variances in basic substitution patterns gives rise to several

groups, with the main subclasses being flava-3-ols, isoflavones, flavanones flavonols, flavones, and anthocyanicidins (**HEIM et al., 2002**). Chemical and structural assortment of flavonoids is related to their varied properties and functions in plant protection from radiation of the sun, herbivores and invading pathogens (**HARBORNE and WILLIAMS, 2000**). Environmental factors seem to add significantly to the content of phenolic acids and flavonoids in plants. Anthocyanin co-pigments in flowers serve to attract pollinating insects (**HARBORNE and WILLIAMS, 2000**) and are accountable for the blue colours of berries, wines and vegetables all of which are chief bases of flavonoids in the human diet (**CARANDO et al., 1999; STEWART et al., 2000; LOPEZ et al., 2001**).

A main purposes of flavonoids in plants is their defensive character against microbial invasion (**WATERMAN and MOLE, 1994; HARBORNE and WILLIAMS, 2000**). The presence of flavonoids as phytoalexins are important in response to microbial attack (**GRAYER et al., 1994; HARBORNE, 1999**). Much research on flavonoids in the pharmaceutical and pharmacological areas report antitumor, anti-hepatotoxic, anti-lipolytic, vasodilator, antimicrobial, anti-inflammatory, antioxidant, immunostimulant and antiallergic agents (**IINUMA et al., 1994; ALIAS et al., 1995; WILLIAMS et al., 2013; BURDA and OLESZEK, 2001**). HIV research is also considering the role of plant flavonoids as potential drug agents for the immunodeficiency virus as many traditional healers claim medicinal plant treatment and healing for HIV/AIDS (**HARBORNE and WILLIAMS, 2000**).

Tannins are phenolic compounds that display multifaceted and exceedingly variable chemical arrangements. They are broadly characterised into hydrolysable and condensed tannins, based on whether acids or enzymes can hydrolyse the components or whether they condense the components to polymers (**SCHOFIELD et al., 2001**). Both groups of tannins are abundant in highly reactive hydroxyl groups occurring from the benzene rings, and they form multiplexes with proteins including enzymes (**WALLACE and FRY, 1994**) and polymers such as cellulose and hemicellulose (**HASLAM, 1989**). Hydrolysable tannins are based upon the fundamental structural unit of gallic acid (3, 4, 5-trihydroxyl benzoic acid) and are almost invariably found as multiple esters with D-glucose to form gallotannins (**HASLAM, 1989**). By-products of hexahydroxydiphenic acid (ellagitannins) are derived from oxidative coupling of adjacent galloyl ester groups in a polygalloyl D-glucose ester (**MUELLER-HARVEY, 2001**). The fundamental compound, pentagalloylglucose is the start of various tannin structures. The compound consists of polyols such as glucose, surrounded by several gallic acid units. Gallic acid and its metabolites are widely distributed in plants, particularly

herbaceous dicotyledons (MUELLER-HARVEY, 2001). In plant tissues like galls, metabolites of gallic acids accumulate in large quantities (HASLAM, 1989).

Proanthocyanidins or condensed tannins are oligomers of 3-flavonols (catechins) and 3, 4 flavan-diols (leucoanthocyanidins) connected together by single interflavan carbon to carbon bonds (HAGERMAN, 1998; XIE and DIXON, 2005). The flavan-3-ol units are interconnected principally through the 4 and 8 positions. The term proanthocyanidin is a derivative of the acid-catalysed oxidation reaction that yields red anthocyanidins upon heating in an acidic alcohol solution (PORTER et al., 1986). Reactions that form the basis of the butanol-HCl assay for proanthocyanidins. Their arrangements are subject to the nature (hydroxylation and stereochemistry pattern) of the flavan-3-ol starter and location, the degree of polymerisation and the presence of modifications such as esterification of the 3-hydroxyl group extension units and stereochemistry of the linkage lower unit. Most proanthocyanidins are built from catechin, epicatechin and flavan-3-ols, (XIE and DIXON, 2005). They are widespread polyphenols in plants after lignins and occur in high concentrations in various plant parts (MATTHEWS et al., 1997). In plants, condensed tannins act as feeding deterrents in reproductive tissues and developing fruit and also impart astringency to fruit and fruit products (HASLAM et al., 1989). According to FEENY (1970) tannins are characteristic of the chemical defence of plants and act as quantifiable dosage dependent barricades to marauders that feed on plants. The effects physiologically of tannins on predation is assumed to derive from their ability to combine with proteins (SCHULTZ et al., 1992; HASLAM, 1996). VAN DEN BERGHE et al. (1985) reported that tannins are responsible for binding to the virus and/or protein of the host cell membrane and affect adsorption of the virus and similarly in fungal and bacterial enzymes that are bound by tannins (SCHULTZ et al., 1992). The antibiotic, biostatic and antioxidant effects on a variety of microorganisms that ingest them (HASLAM, 1989) have several medicinal properties (HASLAM, 1989; OKUDA et al., 1992; GURIB-FAKIM, 2006).

**Table 3.1** Total phenolic, flavonoid and proanthocyanidin content of the nineteen extracts from the screened medicinal plants

Plant species	Plant part	Total phenolics (mg GAE/ g DW)	Flavonoid content (mg CTE/g DW)	Proanthocyanidin content ( $\mu$ g LCE/g DW)
<i>Aloe</i>				
<i>arborescens</i>	Leaves	<b>*4.69 <math>\pm</math> 0.07</b>	<b>*3.41 <math>\pm</math> 0.30</b>	1.80 $\pm$ 0.02
<i>Aloe aristata</i>	Leaves	<b>*4.53 <math>\pm</math> 0.09</b>	<b>*3.43 <math>\pm</math> 0.22</b>	<b>*3.51 <math>\pm</math> 0.83</b>
<i>Aloe ferox</i>	Leaves	<b>*4.64 <math>\pm</math> 0.10</b>	<b>*5.28 <math>\pm</math> 0.80</b>	<b>*5.28 <math>\pm</math> 0.25</b>
<i>Bulbine</i>				
<i>frutescens</i>	Leaves	0.29 $\pm$ 0.01	0.66 $\pm$ 0.11	0.34 $\pm$ 0.04
	Bulbs	0.70 $\pm$ 0.03	0.64 $\pm$ 0.03	0.37 $\pm$ 0.08
<i>Bulbine</i>				
<i>natalensis</i>	Leaves	0.22 $\pm$ 0.04	0.23 $\pm$ 0.04	0.28 $\pm$ 0.06
	Bulbs	1.19 $\pm$ 0.11	1.06 $\pm$ 0.08	1.89 $\pm$ 0.07
<i>Eucomis</i>				
<i>autumnalis</i>	Leaves	1.61 $\pm$ 0.09	1.34 $\pm$ 0.03	0.65 $\pm$ 0.11
	Roots	0.15 $\pm$ 0.06	0.14 $\pm$ 0.03	0.19 $\pm$ 0.01
	Bulbs	0.53 $\pm$ 0.02	1.21 $\pm$ 0.01	0.90 $\pm$ 0.05
<i>Haworthia</i>				
<i>limifolia</i>	Leaves	<b>*4.46 <math>\pm</math> 0.10</b>	<b>*3.11 <math>\pm</math> 0.84</b>	<b>*3.04 <math>\pm</math> 0.25</b>
<i>Hypericum</i>				
<i>aethiopicum</i>	Leaves	<b>*3.35 <math>\pm</math> 0.04</b>	<b>*4.56 <math>\pm</math> 0.30</b>	<b>*9.85 <math>\pm</math> 0.20</b>
<i>Merwillia</i>				
<i>plumbea</i>	Leaves	0.80 $\pm$ 0.06	1.01 $\pm$ 0.04	0.45 $\pm$ 0.01
	Bulbs	0.59 $\pm$ 0.02	0.78 $\pm$ 0.07	<b>*2.04 <math>\pm</math> 1.75</b>
	Roots	0.05 $\pm$ 0.02	0.45 $\pm$ 0.13	0.37 $\pm$ 0.08
<i>Tetradenia</i>				
<i>riparia</i>	Leaves	1.39 $\pm$ 0.10	0.89 $\pm$ 0.04	0.43 $\pm$ 0.05
	Stems	0.56 $\pm$ 0.09	0.27 $\pm$ 0.05	0.09 $\pm$ 0.02
<i>Zantedeschia</i>				
<i>aethiopica</i>	Leaves	0.56 $\pm$ 0.03	<b>*2.13 <math>\pm</math> 0.04</b>	0.61 $\pm$ 0.02
	Stems	0.16 $\pm$ 0.01	0.63 $\pm$ 0.06	0.24 $\pm$ 0.00

Each value in the table represent mean  $\pm$  standard error (n = 3). \*Bold indicates the significant plant extracts for total phenolics, flavonoid content and proanthocyanidin content.

### 3.4 Conclusions

The production of phenolic compounds in the eleven plant species and their plant parts were analysed in this study. Understanding the phytochemical concentrations in each medicinal plant species will help medicinal plant research and users to better understand the beneficial and harmful compounds and their effective concentration levels. This will guide users when harvesting plant parts rather the whole plant as an important conservation practice to eliminate the extinction of these commonly used medicinal plants. Total phenolics, flavonoids and proanthocyanidins were determined for the selected plants and some positive results were obtained which suggest their roles as anti-inflammatory and antioxidant agents in the processes essential for the management of wound healing and dermatological diseases from medicinal plants. Further research on the identification and isolation of bioactive compounds responsible for these effects is necessary as plants contain multi-complex secondary metabolites which may have synergistic effects and not function independently of each other. Through scientific investigations the key to unlock these compounds and their exact functions will be a significant contribution to traditional public health care.

In the phytochemical assessment for total phenolics, flavonoids and proanthocyanidins of the screened medicinal plants; seven of the plants stood out with results that scored high content of phytochemical compounds. These are the *Aloe* species across all three assays (4.53±0.09 to 4.69±0.07 mg GAE/gDW for total phenolics; 3.41±0.30 to 5.28±0.80 mg CTE/gDW for flavonoid content; 3.51±0.83 to 5.28±0.25 µg LCE/gDW for proanthocyanidin content), *Haworthia* (4.46±0.10 mg GAE/gDW for total phenolics, 3.11±0.84 mg CTE/gDW for flavonoid content, 3.04±0.25 µg LCE/gDW for proanthocyanidin content) and *Hypericum* (3.35±0.04 mg GAE/gDW for total phenolics, 4.56±0.30 mg CTE/gDW for flavonoid content, 9.85±0.20 µg LCE/gDW for proanthocyanidin content), *Merwillia* 2.04±1.75 µg LCE/gDW for proanthocyanidin content and *Zantedeschia* 2.13±0.04 mg CTE/gDW for flavonoid content.

# CHAPTER FOUR

## CYTOTOXICITY

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### 4.1 Introduction

The safety and efficacy of traditional medicines for wound healing and skin disorders of plant extracts are of high importance and raises considerable concerns for their applicability and use on humans. Plants frequently used in indigenous medicinal systems are assumed to be safe. Recent scientific evidence shows that many plants are potentially toxic, mutagenic and carcinogenic (KASSIE et al., 1996). Many assays that are biological need a measurement of living and/or flourishing mammalian cells. Treating human cells with cytotoxic compounds present in plant extracts can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can stop actively growing and dividing (a decrease in cell viability), or can activate a genetic program of cell death (apoptosis) (KASSIE et al., 1996). Cells that undergo rapid necrosis *in vitro* do not have sufficient time or energy to activate the apoptotic machinery. Cells in cultures undergoing apoptosis eventually undergo secondary necrosis. They shut down metabolism, lose membrane integrity and lyse. It is therefore prudent to measure cytotoxicity and cell viability effects of medicinal plant extracts (KASSIE et al., 1996). Many active plant components are toxic at elevated doses and thus an approach to carry out effective screening at different concentrations is essential. Brine shrimp is a convenient probe for pharmacologic activities in plant extracts which may be manifested as toxicity towards the newly hatched brine shrimp.

Various methods are available for screening the safety of plant extracts, two of which were applied in the present study to evaluate the cytotoxicity of the eleven traditional medicinal plant extracts under investigation. Brine shrimp lethality bioassay is based on the ability to kill laboratory cultured brine shrimp (*Artemia nauplii*). The assay serves as a preliminary assessment of toxicity and has been used for detecting fungal toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity testing of various materials used by humans (HARWING and SCOTT, 1971; McLAUGHLIN et al., 1991; MARTINEZ et al., 1998; BARAHONA and SANCHEZ-FORTUN, 1999; PELKA et al., 2000). A study by HOSSAIN et al. (2012) reports on the use of leaves of *Caloptropsis gigantean*, used

traditionally for the external application of eczema, skin eruptions, old sores and ulcers. The LC<sub>50</sub> values or concentrations at which 50% death rates of brine shrimp occurred were reported. A review by **FENNELL et al. (2004)**, reports an assessment of African medicinal plants used for various illnesses focusing on their efficacy and safety, where poisoning from traditional medicines were indicated; and this occurred frequently because the plants were misidentified when sold, or preparation and administration by incompetently trained personnel was erroneous.

The tetrazolium-based colorimetric assay 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT), has several favourable characteristics for assaying cell proliferation and survival. This assay measures the number and activity of living cells at the end of the assay (**KAPPLER et al., 1981**). MTT is tested and known to be cleaved by all living, metabolically active cells. The amount of formazan generated is directly proportional to the cell numbers in a homogeneous cell population. Activated cells produce more formazan than resting cells, which could allow the measurement of activation even in the absence of proliferation. These properties are all consistent with the cleavage of MTT only by active mitochondria. The main advantage of the colorimetric assay is the speed with which samples can be processed. The substrate does not interfere with measurement of the product. This allows the assay to be read with no removal or washing steps, which increases the speed of the assay and helps to minimize variability between samples. The ultimate phases of the assay namely adding the MTT, reading the plates and printing of the data takes minimal time compared to the setting up of the assay which includes growth factor dilutions and mixing cells. The assay can be read minutes after the addition of acid-isopropanol and the colour is constant for a few hours at room temperature. The results are also clearly seen visually. According to **FENNELL et al. (2004)**, genotoxicity research revealed that multiple medicinal plants are responsible for detrimental effects to the genetic components and should be used cautiously. **DU PLOOY et al. (2001)** reported a study on *Boophane disticha* scales that are used for dressing circumcision wounds and also used in oral concoctions. The effects on the human body include sedation, analgesic effects, visual hallucinations, irrational behaviour, coma and/or death. The objectives of this study were to evaluate the cytotoxicity properties of the extracts of the selected plants using Brine shrimp and MTT assays.

## 4.2 Material and Methods

### 4.2.1 Brine Shrimp Assay

The Brine shrimp assay (MEYER et al., 1982) was used to determine the extract toxicity of *Artemia salina*. Brine shrimp eggs (Ocean Nutrition™ PET 152) obtained from Northlands Pets, Durban, South Africa were hatched under light in a 500 ml beaker containing sea water for 36 - 48 h. The eggs were ready once swimming Brine shrimp nauplii had hatched. Two mg/ml of each plant extract were two-fold serially diluted into five concentrations (2 mg/ml; 1 mg/ml; 0.5 mg/ml; 0.25 mg/ml and 0.125 mg/ml). Ten to 15 live Brine shrimps were transferred from the hatching beaker to each test tube using a Pasteur pipette with sea water and the water topped up to 10 ml with sea water. This was done in triplicate for every diluted plant extract. Each plant extract (100 µl) was added to the test tubes and allowed to stand at room temperature for 24 h. DMSO and an organophosphate served as negative and positive controls respectively. For the plant extract to be lethal, shrimp death of greater than 50% had to be recorded after 24 h.

### 4.2.2 MTT colorimetric assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

The method described by MOSMANN (1983) was used to determine the cytotoxicity of the extracts. The plant extracts were tested for cytotoxicity against African green monkey Vero kidney cells obtained from the Department of Veterinary Tropical Diseases (University of Pretoria). The cells were maintained in Minimal Essential Medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamycin (Virbac) and 5% foetal calf serum (Highveld Biological). Cell suspensions were prepared from confluent monolayer cultures and plated at a density of  $5 \times 10^4$  cells into each well of a sterile 96-well microtitre plate. Plates were incubated for 24 h at 37 °C in a 5% CO<sub>2</sub> incubator until the cells were in an exponential phase of growth and the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the plant extracts (200 µl) were prepared by serial dilutions in ethanol and prepared in MEM and added to the cells. The viable cell growth after 120 h incubation with plant extracts was determined using the tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT), (Sigma) described by MOSMANN (1983). Untreated cells and a positive control (Doxorubicin chloride, Pfizer Laboratories) were included. The amount of MTT reduction was measured immediately by detecting absorbance using a



Chromate 4300 microplate reader at 540 nm and a reference wavelength of 630 nm. The wells in column 1, containing medium and MTT but no cells, were used to standardize the plate reader. The LC<sub>50</sub> values were calculated as the concentration of test compound resulting in a 50% reduction of absorbance as compared to untreated cells. The intensity of colour was directly proportional to the number of surviving cells and viable cell growth. Tests were carried out in quadruplicate and each experiment was repeated three times.

### 4.3 Results and Discussion

In addition to evaluating the plant extracts for pharmacological properties, it is as important to ascertain the safety of medicinal plant extracts for human use. Cytotoxicity assays are some of the important tools for this purpose as they shed light on the potential toxic or mutagenic effects of plant extracts. Toxicity effects of most medicinal plant extracts are not well documented largely because of the widely held belief that traditional medicinal plants are safe due to continuous use over long periods (ELGORASHI et al., 2003). An evaluation of the toxicity and cytotoxicity are an important part of ethnopharmacological validations (VAN DYK et al., 2009). The severity of toxicity of extracts are often related to concentration, exposure time and physiological parameters (MEYER et al., 1982). The Brine shrimp assay determines the relationship between brine shrimp lethality and cytotoxicity and a positive correlation exists between brine shrimp lethality and cytotoxicity (AYO et al., 2007). The MTT assay on the other hand evaluates the survival and proliferation of mammalian cells upon exposure to the extracts. The results of the plant extracts tested in this study using the Brine shrimp lethality assay (Table 4.1) indicated lack of cytotoxicity after 24 h. For an extract to be considered lethal it must demonstrate more than 50% Brine shrimp death. The effectiveness of plant extracts is often measured as LC<sub>50</sub>, which represents the concentration of the extract that is able to affect 50% cell death (cytotoxicity). The MTT assay further substantiated the Brine shrimp results with LC<sub>50</sub> values of >1 mg/ml for all extracts (Table 4.1). An LC<sub>50</sub> value of less than 0.5 mg/ml for an extract would be of cytotoxic concern in this assay while that of below 0.1 mg/ml is considered highly cytotoxic. The results of the cytotoxicity assay on the tested extracts show safe levels, however, further mutagenic and toxicological tests with different mechanisms of action and targets would be necessary to ascertain the complete safety of these plant extracts.

**Table 4.1** Brine Shrimp Lethality and Cytotoxicity effects (MTT assay) of the different plant extracts used in this study

Plants extract	LC <sub>50</sub> (mg/ml) Brine shrimp lethality	LC <sub>50</sub> (mg/ml) MTT assay
<i>Aloe arborescens</i> leaves	>2	>1
<i>Aloe aristata</i> leaves	>2	>1
<i>Aloe ferox</i> leaves	1.93	>1
<i>Bulbine frutescens</i> leaves	>2	>1
<i>Bulbine frutescens</i> bulbs	>2	>1
<i>Bulbine natalensis</i> leaves	>2	>1
<i>Bulbine natalensis</i> bulbs	>2	>1
<i>Eucomis autumnalis</i> leaves	>2	>1
<i>Eucomis autumnalis</i> bulbs	>2	>1
<i>Eucomis autumnalis</i> roots	>2	>1
<i>Haworthia limifolia</i> leaves	>2	>1
<i>H. aethiopica</i> leaves	>2	>1
<i>Merwillia plumbea</i> leaves	>2	>1
<i>Merwillia plumbea</i> bulbs	>2	>1
<i>Merwillia plumbea</i> roots	>2	>1
<i>Tetradenia riparia</i> leaves	>2	>1
<i>Tetradenia riparia</i> stems	>2	>1
<i>Zantedeschia aethiopica</i> leaves	>2	>1
<i>Zantedeschia aethiopica</i> stems	>2	>1
DMSO	>2	
Organophosphate	-	Doxorubicin LC <sub>50</sub> (uM) 4.654 ± 0.971

The cytotoxicity results (LC<sub>50</sub>) of the plant extracts were all greater than 2 mg/ml except for *Aloe ferox* leaves in the Brine shrimp assay. The cytotoxicity results of the plant extracts at all concentrations were greater than 2 except for *Aloe ferox* leaves in the Brine shrimp assay. Extracts considered lethal would demonstrate greater than half shrimp death. The MTT cytotoxicity test yielded LC<sub>50</sub> values of > 1 mg/ml on all extracts indicating that they are not cytotoxic. The observed general lack of cytotoxic effects on all the tested extracts presents some promising and beneficial aspects of these medicinal plant extracts in the treatment of skin diseases and thus in potential for wound healing. The two *Aloe* species and *H. aethiopicum*

were among the best extracts that exhibited consistently good antimicrobial activity and warrant further investigations and possible isolation of bioactive principles. This would require further investigations into the application into potential bioactive compounds for dermatological use.

Cytotoxicity may result in genotoxicity and mutagenicity of cells. The induction of permanent transmissible variations in the assembly of the genetic material in cells or organisms is known as mutagenicity. These alterations or mutations may involve a single gene or a block of genes. Genotoxicity is comprehensive and entails the ability of the compounds to interact with the DNA and cellular apparatus such as topoisomerase enzymes and spindle apparatus which regulates the fidelity of the genome. Isolated compounds from plants like quercetin, fluoroquinolone alkaloids and isothiocyanates are considered to be mutagens. Isolating compounds that are responsible for mutagenic effects detected with plant extracts are complex due to the mixture of organic compounds (SRIVIDYA et al., 2012). Therefore, safety of plants and their continued extensive use in health care is very important. There are studies that have confirmed that some phytochemicals might possess anti-mutagenicity and anticarcinogenic effects. The intake of plant materials in various epidemiological studies also support the chemo-preventive effects in which plant chemicals display genotoxic/mutagenic effects by themselves or perpetuate the effect of various xenobiotics (RANI et al., 2005). Multifaceted chronic disease pathology of cancer, cardiovascular disease, diabetes, hypertension, immune and neuro-degenerative disorders and the ageing process as well as skin conditions and wound healing can be modulated by polyphenols derived from plants. Oxidative stress occurs in cells and tissues which lead to human disease when the ROS generated exceeds the antioxidant capability causing toxic shock. Protective antioxidant plant derivatives are reported due to radical scavenging, inhibition of oxidase, alteration of gene expression and metal chelation to the antioxidant response elements and various multidimensional effects of phenolic compounds. VAN ZYL and VILJOEN (2002) found limited data on antiplasmodial activity and *Aloe* species toxicity. VAN DYK et al. (2009) reported the importance of including assays for toxicity when screening; they used fifty-nine different plant extracts from six plant species and found that only one extract isolated from *Combretum erythrophyllum* showed a favourable safety index of 0.03 µg/ml.

#### **4.4 Conclusions**

Cytotoxicity screening on all plant extracts for both Brine shrimp lethality and the MTT assay exhibited no cell toxicity effects having  $LC_{50}$  values larger than two mg/ml and greater than one mg/ml respectively. However, further investigations using different models and mechanisms are necessary as many of the selected medicinal plants are reported to be toxic and misuse may result in mortality.

## CHAPTER FIVE

### ANTI-INFLAMMATORY ACTIVITY:

#### *IN VITRO* NITRIC OXIDE (NO) AND 5-LIPOXYGENASE INHIBITION (LOX)

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##### 5.1 Introduction

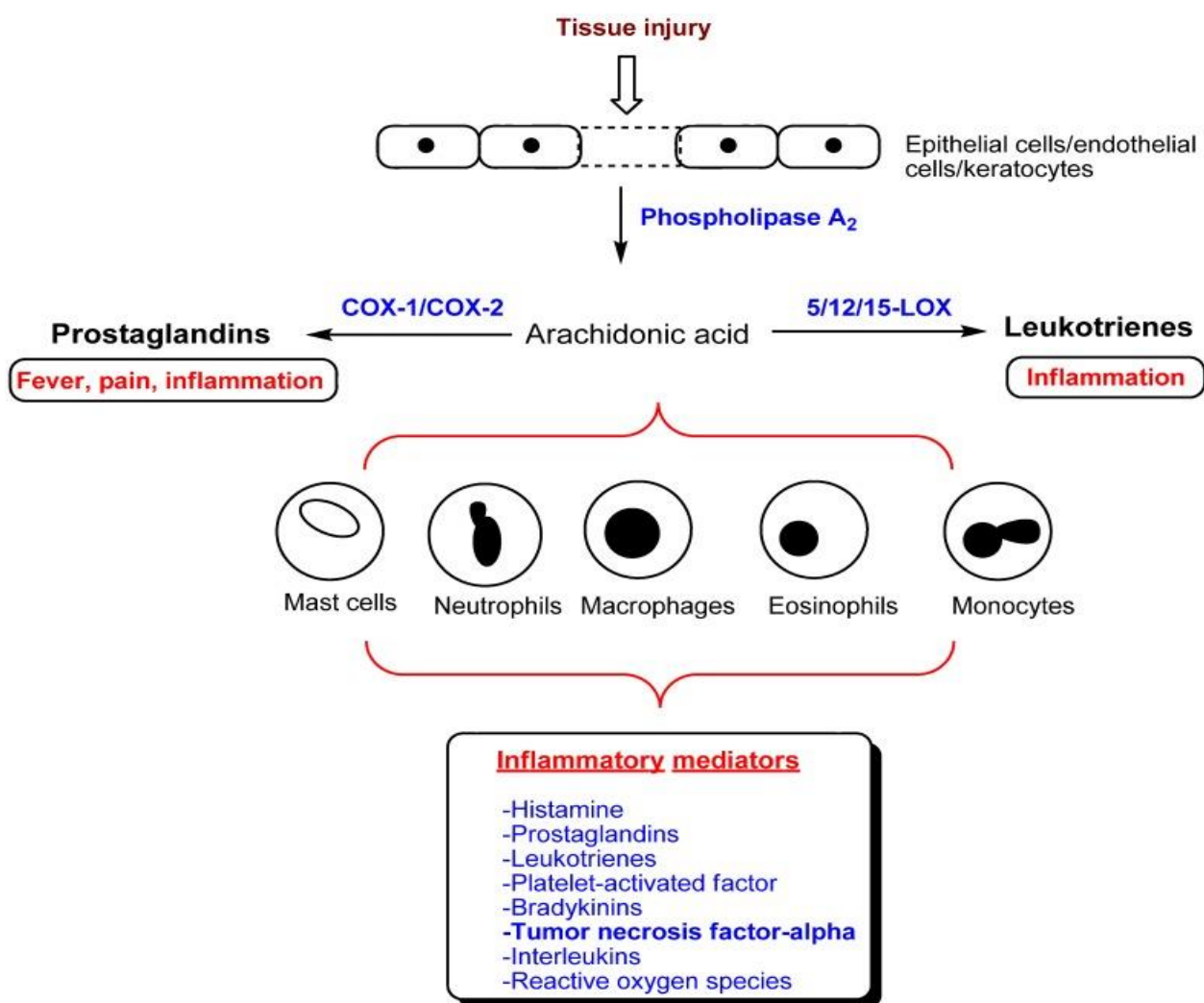
The mammalian immune system is comprised of many interactive, specific types of cells that cooperatively safeguard the mammalian body from pathogenic infections and other sources of stimuli. Chronic inflammation is pivotal to skin pathologies and delayed healing of wounds in diseases like leprosy and sexually transmitted infections (**ALLEN, 2003**). According to **IWALEWA et al. (2007)**, plants do contain an extensive array of natural compounds which have effects on the healing process and the potential to inhibit or reduce the inflammatory process. A study conducted by **JOHNSON (2009)** emphasised the importance of research in alleviating the lack of alternative drugs, the promotion and the development of new drugs as well as the management of therapeutic processes like inflammation and related metabolic conditions. **EDWARDS (2005)** indicated that the process of inflammation is a huge burden to human health care and involves many diseases. Even though there are multiple drugs available, many of the drugs that are anti-inflammatory come with extensive side-effects and have limited clinical use (**VIOLA et al., 2008**). The demand for safe and effective drugs that are anti-inflammatory is essential for the future of dermatological and wound healing care.

Inflammation can be defined as a localized protective, non-specific immune response of vascular tissues to harmful stimuli such as damaged cells or irritants and pathogens (**FERRERO-MILIANI et al., 2007**). Inflammation is a series of initial responses from the immune system to infection transferring immune cells to the site of injury. In addition, inflammatory reactions serve to establish a physiological barrier against the spread of infection and to promote healing of any damaged tissue following the clearance of the stimuli (**COHN and LANGMAN, 1996**). A defence mechanism of inflammation is evoked by body tissues in response to chemical perturbation and microbial infection that results in cell injury or death (**O'BYRNE et al., 2000; O'BYRNE and DALGLEISH, 2001**). The inflammation mechanisms involve various events involving the metabolism of arachidonic acid which has

an important role. The defensive response is characterised on the skin as redness, pain, heat and swelling resulting in a loss of function in the injured area. When tissue cells become injured they release kinins, prostaglandins and histamine (**O'BYRNE et al., 2000; O'BYRNE and DALGLEISH, 2001**). Collectively, these compounds increase vasodilation and permeability of the capillaries leading to increased blood flow to the injured site. Short term inflammation is the early reaction of the body to detrimental stimuli and is an outcome of the amplified movement of plasma and leukocytes especially granulocytes from the blood into injured tissues (**O'BYRNE et al., 2000, O'BYRNE and DALGLEISH, 2001**). Numerous biochemical events unfold, increasing the inflammatory response. These include the immune system, various cells within the injured tissues and the local vascular system. Long term inflammation occurs with an advanced shift in the type of cells present in the site of inflammation characterised by simultaneous destruction and healing of the tissue from the inflammatory process (**TORTORA et al., 2016**). Prostaglandins and leukotrienes are produced from arachidonic acid and are responsible for the complex process of inflammation and pain. **JÄGER et al. (1996); FOEGH et al. (1998)** and **GRAF et al. (2005)** reported that flavonoids such as quercetin inhibit lipoxygenase enzymes which impact on pro-inflammatory prostaglandins and leukotrienes (Figure 5.1). These are metabolised by the pathway of LOX to hydroperoxy-eicosatetraenoic acids and leukotrienes that are imperative biologically active mediators in a variety of inflammatory processes (**PIPER et al., 1994; ALITONOU et al., 2006**). Stimuli of neutrophils causes the cleavage of arachidonic acid from the membrane phospholipids which are then transformed into leukotrienes and prostaglandins through LOX pathways (**BOURICHE et al., 2005**). Prostaglandins have numerous effects on physiological processes such as muscle relaxation, vasodilation, aggregation of blood platelets, cell protection, pain and fever control (**GRAF et al., 2005; BEDINI et al., 2009**). The processes of inflammation include processes with reactive oxygenation that are initiated by the stimulation of leucocytes.

Myrtle and eucalyptus essential oils caused temporary leucocyte activation by scavenging hydroxyl radicals and consequently interfering with the process of inflammation by acting as antioxidants (**GRABMANN, 2000**). Antioxidant properties also provide significant evidence about the possible activity of a drug on inflammatory processes (**NJENGA and VILJOEN, 2006**). A combination of studies on LOX and antioxidant assays (Chapter 6) is a good sign of the potential ant-inflammatory activity of medicinal plant extracts (**ALITONOU et al., 2006**). Polyphenols are carbon-based aromatic phenyl ring compounds which oxidize to quinones by reactive oxygen species, a characteristic responsible for the antioxidant activity and anti-

inflammatory activity (SREEJAYAN and RAO, 1996). The structural and functional diversity of phytochemicals are the underlying pharmaceutical capacity for inflammatory activity in medicinal plants. The benefits of inflammation are the increasing levels of inflammatory signals simply, it's the body's response to outside threats like stress, infection, or toxic chemicals. When inflammation occurs, chemicals from the body's white blood cells are released into the blood or affected tissues to protect the body from foreign substances. This release of chemicals increases the blood flow to the area of injury or infection, and may result in redness and warmth which ultimately initiates the healing process. Inflammation pathway (Figure 5.1) below and the relationship to selecting LOX and NO inhibition in this study.



**Figure 5.1** Pathway of the Inflammation Process. (PEDERSEN, COSKUN, SOENDERGAARD, SALEM, HAAGEN NIELSEN. 2014).

### 5.1.1 5-Lipoxygenase and the Inflammatory Process

The LOX enzymes are non-haem in nature and contain ferric iron catalytic ability with an increased reduction potential. A study by DAILY and IMMING (1999) report that iron chelators like hydroxamic acid derivatives and catechols are able to reduce iron from active to

its inactive form, preventing the transformation of fatty acids to pro-inflammatory lipid mediators. Compounds like arachidonate 5-lipoxygenase inhibitors reduce or inhibit the activity of the arachidonate 5-LOX enzyme, these are responsible for the production of inflammatory leukotrienes. The excessive production of leukotrienes is a chief consequence of inflammation in allergic rhinitis, osteoarthritis and asthma (**LAUFER, 2003; NELSON and COX, 2008**). Examples of 5-LOX inhibitors include drugs such as zileuton and sodium meclofenamate (**BISHAYEE and KHUDA-BUKHSH, 2013**).

A wide variety of agents have been reported as 5-LOX inhibitors with the majority appearing to be reducing agents that are lipophilic including partially saturated aromatics, phenols and compounds with heteroatom-heteroatom bonds. Many are not selective 5-LOX inhibitors, but often affect COX and other LOXs as well. *In vivo* systemic activity for many of these has in general been disappointing, probably because of poor bioavailability caused by lipophilicity and metabolic instability (oxidation, and conjugation of phenolic compounds). However, topically a number of agents have shown promise for skin inflammation, with Syntex's lonapalene being the most advanced of these (**BISHAYEE and KHUDA-BUKHSH, 2013**). Many results published to date appear more disappointing in the allergy/asthma field. More excitingly, a few structural types of anti-inflammatory agents are selective 5-LOX inhibitors which have shown systemic activity *in vivo* in some clinical studies. There are certain chemicals occurring in trace amounts of food products, and in dietary supplements shown to inhibit 5-LOX; these include caffeic acid, curcumin and baicalein (**BISHAYEE and KHUDA-BUKHSH, 2013; DE MELO et al., 2014; WÖLFLE et al., 2014**).

LOX enzymes are an iron-containing family of enzymes many of which catalyse the deoxygenation of polyunsaturated fatty acids in lipids with a cis,cis-1,4- pentadiene structure (**CHARLIER and MICHAUX, 2003; FOURIE, 2009; POECKEL and FUNK, 2010**). These plant enzymes are most common and are involved in various diverse aspects of plant physiology in relation to pest resistance, senescence or responses to wounding growth and development. Mammalian tissues show that a number of lipoxygenase isozymes are involved in the production of eicosanoids (such as leukotrienes, nonclassic eicosanoids and prostaglandins) (**CHARLIER and MICHAUX, 2003; FOURIE, 2009; POECKEL and FUNK, 2010**).

The LOX enzymes are successful in the management of diseases like atherosclerosis, cancer, asthma and inflammation (**CHARLIER and MICHAUX, 2003; FOURIE, 2009;**



**POECKEL and FUNK, 2010**). Studies report the blocking of arachidonic acid (AA) metabolism with LOX inhibition leading to generating pro-inflammatory leukotrienes and lipoxins in the LOX pathway. These intermediary products are reported to be the cause of the side effects of inhibitors. A number of small molecule COX/LOX inhibitors are known to overcome this. **REDDY et al. (2008)**, reported on indolylpyrazoline agents as dual COX/LOX inhibitors but with no *in vivo* anti-inflammatory activities. A study using virtual selection to recognize ring frameworks as double COX/LOX inhibitors showed that a thiazolidinone ring framework may be successfully used for developing anti-inflammatory agents which would be a significant discovery and progress for medicinal plant compounds (**GERONIKAKI, 2008**). Many hybrid COX/LOX inhibitors have an *N*-difluoromethyl-1, 2-dihydropyridine-2-1 LOX pharmacophore (**CHOWDHURY et al., 2009; YU et al., 2010**). In another study, virtual screening identified a based ring framework benzo-dithiazoliumylide as double COX/5-LOX inhibitor (**YU et al., 2010**). No reports or information for these agents has been presented of *in vivo* anti-inflammatory activity.

### **5.1.2 Nitric Oxide as an Anti-Inflammatory Agent**

Nitric oxide (NO) is one of many oxides of nitrogen, a colourless gas, a free radical with an unpaired electron on the nitrogen atom. It is a heteronuclear diatomic molecule. The molecules may be produced during fossil fuel combustion at power plants and by automobile engines. Excess NO in high air temperatures is needed for combustion of the fuel. Natural production occurs by high air temperatures produced during lightning in thunderstorms (**SHARMA et al., 2006; SHARMA et al., 2007**). NO is a signalling molecule with an important function in inflammation pathogenesis and provides an anti-inflammatory effect in normal physiological conditions. Nitric oxide is an inflammatory mediator that induces inflammation as it is overproduced in irregular physiological circumstances (**SHARMA et al., 2007**). When exercising, the muscles require higher levels of oxygen which are supplied by the blood. When the heart drives blood under pressure the lining in the arteries releases nitric oxide into the blood which relaxes and widens the walls of the blood vessels permitting blood to flow through freely. The production of NO allows the release into the endothelial cells which convert arginine into citrulline (**SHARMA et al., 2007; RAO et al., 2010**). NO's main function is to improve bactericidal and tumoricidal activity of macrophages (**STUEHR and NATHAN, 1989; NATHAN and HIBBS, 1991**). It induces vascular dilatation in the heart and blood vessels and is further involved in immunity through cytokine-activated macrophages, releasing NO in elevated concentrations. Researchers have reported that NO is a powerful neurotransmitter effectively at the neuron synapses, contributing to apoptosis regulation and is

involved in inflammatory pathogenesis of disorders in joints, the gastrointestinal and respiratory systems (**SHARMA et al., 2007; RAO et al., 2010**). NO inhibitors are effective in the management of inflammatory diseases. Arginine analogues and discerning biosynthesis inhibitors of NO are known to reduce inflammation. Excessive production could lead to tissue damage, vasoconstriction and stimulation of mediators for inflammatory activity (**SHARMA et al., 2007; RAO et al., 2010**).

In all mammals, NO is a cell-signalling molecule and a part of physiological and pathological processes and a strong vasodilator. Pharmaceuticals like nitro-glycerine and amyl nitrite are commonly used in wound healing and are precursors to nitric oxide (**SHARMA et al., 2007; RAO et al., 2010**). Small quantities of nitric oxide protect organs like the liver from ischemic damage. NO production is involved in non-alcoholic liver fatty disease and is important in lipid hepatic or liver fatty acid metabolism under malnourishment (**SHARMA et al., 2007; RAO et al., 2010**).

Nitric acid production is increased in those populations residing at high altitudes, and this assists these people in avoiding hypoxia by increasing pulmonary vasculature vasodilation. Some of the effects known to occur from high NO levels include neurotransmission modulation of the hair cycle, production of reactive nitrogen intermediates vasodilatation, and penile erections (through its ability to vasodilate) (**STUEHR and NATHAN, 1989; NATHAN and HIBBS, 1991**). Amyl nitrite and nitro-glycerine serve as vasodilators as they are transformed into NO in the body. The antihypertensive drug Minoxidil induces vasodilating and comprises an NO moiety and exhibits itself as an NO agonist. NO is known to obstruct and inhibit smooth muscle growth and contraction, leukocyte adhesion and platelet aggregation to the endothelium (**STUEHR and NATHAN, 1989; NATHAN and HIBBS, 1991**). Hypertension, atherosclerosis and diabetes physiology demonstrated compromised NO pathways. High salt intake increases NO production in patients with hypertension (**STUEHR and NATHAN, 1989; NATHAN and HIBBS, 1991**).

The NO synthesis pathway in phagocytes generates high levels of NO that induces apoptosis. Studies conducted *in vitro* have reported that phagocyte-dependent NO production at applications higher than 400-500 mg/ml induces cellular apoptosis. The effect is like mediators that reverse inflammatory responses by neutralizing and clearing of inflammatory cells from inflamed tissues (**STUEHR and NATHAN, 1989; NATHAN and HIBBS, 1991**). NO's role

in inflammation is not simple, signifying that the gaseous mediator can also encourage inflammation (**STUEHR and NATHAN, 1989; NATHAN and HIBBS, 1991**).

Bacterial pathogens have developed mechanisms for NO resistance (**RAO et al., 2010**). Nitric acid serves as a measure of inflammation in conditions like asthma. Nitric oxide can contribute to perfusion injury by interacting with a superoxide to form detrimental oxidant peroxynitrite. Inhaled nitric oxide helps survival and recovery from a chemical called paraquat which causes poisoning, causing lung tissue-damaging superoxides and interferes with NOS metabolism (**RAO et al., 2010**). In plants, it is a signalling molecule, acting against stress caused by oxidation and is active in plant pathogen collaborations. The treatment of cut flowers and plants producing NO is known to result in reducing time to wilting and the delay in senescence (**RAO et al., 2010**).

Investigations into medicinal plant material that can be used as natural inflammatory mediators of inhibitors and enzymes like LOX and NO may be alternatives to the detection of original healing agents against inflammatory diseases (**HARVEY, 2000**). The present study evaluated selected plant extracts using anti-inflammatory LOX activity and NO production inhibition.

### **5.1.3 LOX and NO Enzymes in Inflammation**

LOX (lipoxygenase) and NO synthase enzymes are accountable for the manufacture of prostaglandin H<sub>2</sub> (PGH<sub>2</sub>). The expression of the two LOX isozymes is differently regulated but the enzymes are moderately similar in their amino acid sequences (**FLETCHER et al., 1992**). The constitutive enzyme, LOX, encoded by a 2.8-kilobase transcript located on human chromosome 9, serves certain physiologic housekeeping functions, such as thrombogenic thromboxane A<sub>2</sub> generation by platelets, antithrombogenic PGI<sub>2</sub> production by endothelial cells, generation of cytoprotective prostanoids in the gastric mucosa, and regulation of renal blood flow by prostanoids (**FLETCHER et al., 1992; MURAKAMI et al., 2000**). In contrast, expression of the inducible LOX, encoded by a 4.4-kilobase transcript from human chromosome 1, is found almost exclusively in inflamed tissues responding to inflammatory stimuli such as pro-inflammatory cytokines, growth factors, mitosis stimulants, bacterial lipopolysaccharide, and tumour promoters (**FLETCHER et al., 1992**). The view that the two iso-forms of the LOX enzyme facilitate inflammatory processes and physiological processes, respectively, indicates individual pathways of AA metabolism with various advantages to the organism. There are reports that the two enzyme isoforms use different AA pools in response to different cellular stimuli for prostaglandin synthesis (**REDDY and HERSCHMAN, 1994; MURAKAMI et al., 2000**). Lipoxygenases (LOXs) are a ubiquitous family of non-heme iron enzymes involved in the stereo- and regio-specific peroxidation of arachidonic acid (AA) or

linoleic acid (LA), in the presence of molecular oxygen. One clear difference between the lipoxygenases was in their free sulfhydryl group content. The uv-visible absorption spectrum of each native isoenzyme was consistent with expectations for the experimental aromatic amino acid content. All of the isoenzymes contained one non-heme iron atom per molecule of protein (**DRAHEIM, CARROLL, MCNEMAR, DUNHAM, SANDS, FUNK. 1989**).

The main enzymes that have been described include 5, 8, and 15-LOX and they depend on the oxidation site of fatty acids (**PORTA and ROCHA-SOSA, 2002**). The LOX and NO gene has the structure typical of an “immediate-early” gene product that is rapidly up-regulated during inflammation and other pathologic processes (**CROFFORD, 1997; CROFFORD et al., 2000; BOTTING, 2003; 2006**). LOX is thought to contribute to the generation of prostanoids at positions of inflammation (**CROFFORD et al., 2000**). LOX and NO are consistently conveyed in a wide variety of tissues and produces prostanoids involved in the regulation of normal kidney and stomach functions as well as vascular homeostasis (**MORITA et al., 2002**). The concentration of the LOX and NO enzymes fundamentally remains constant, but minor increases in manifestation occurs in response to encouragement from hormones or growth factors (**BOTTING, 2003; BOTTING, 2006**). There are, however, no examples of separate stimulus-specific regulation of LOX and NO in the same cell at a time when both enzymes are expressed and functioning.

#### **5.1.4 Anti-Inflammatory Agents**

Evidence has shown that the LOX and NO enzymes are important in the inflammatory practises. The research focus of these enzymes are drug developments in the treatment of inflammation. **YUAN et al. (2006)** reported that the biosynthesis of prostanoid is inhibited by nonsteroidal anti-inflammatory drugs which are recommended as anti-inflammatory agents and analgesics. The treatment with these nonsteroidal anti-inflammatory drugs prevents the production of prostaglandins and down-regulates inflammation-related pathological symptoms such as pain and swelling. Their mechanism of action, particularly aspirin and ibuprofen includes inhibition of both the LOX and NO enzymes thereby preventing the pathological synthesis of prostaglandins (**MORITA et al., 2002**). Inhibition of LOX may result in the removal of the cytoprotection function, leading to damaging side effects in the gastrointestinal tract (**GILROY et al., 1998**). The use of the drugs resulted in key changes to the pathophysiological responses of the body, which has led to side effects like bleeding, gastric renal ulceration and irritation (**BOTTING, 2003; BOTTING, 2006**). It was later elaborated that these side effects are associated with inhibition of LOX while the anti-inflammatory

activity is due to inhibition of NO (COPELAND, 2005). The negative effects associated with the use of the non-steroidal anti-inflammatory drugs has initiated much interest in research to substitute the use of anti-inflammatory drugs. Selective inhibition of LOX and NO enzymes has been shown to significantly lower the development of gastro toxicity and related side effects (FERRERO-MILIANI et al., 2007). Traditional health care provides extensive resources for exploitation in the discovery of new anti-inflammatory agents. Many indigenous plant-derived natural compounds like alkaloids, tannins, flavonoids, saponins, and essential oils offer activities that are anti-inflammatory (JUST et al., 1998; GURIB-FAKIM, 2006).

A South African study by IWALEWA et al. (2007) reported on greater than 100 plant species within 60 families that were used for the management of pain related inflammatory disorders in mammals. Bioactivity was found to be related to secondary metabolites like phenols, alkaloids, saponins and terpenoids. Therapeutic and biological properties associated with these metabolites include antimicrobial, anticancer, antioxidant and anti-inflammatory activities (HODZIC et al., 2009). The mechanism of action of diverse compounds of a phenolic nature such as curcumins, flavonoids and tannins and occur through activities of free radical scavenging and inflammatory enzyme inhibition like COX and LOX inflammatory cascades (LEE and MOON, 2003; SPEED and BLAIR, 2011).

Plant-derived products are important in the advancement of developing anti-inflammatory agents. In this study, different plant parts of eleven medicinal plants were evaluated for their inhibition of LOX and NO enzymes. The study aimed to determine and provide evidence if the selected medicinal plant extracts used for dermatological management have anti-inflammatory activity as part of their mechanism of action.

## **5.2 Materials and Methods**

### **5.2.1 Collection and Preparation of Plant Material**

Plant material were prepared as described in Chapter 2, Sections 2.4.1 and 2.4.2.

### **5.2.2 The Nitric oxide (NO) Production and Cytotoxicity of LPS-activated RAW 264.7 Macrophages**

#### **5.2.2.1 Cell Culture Preparation**

The RAW 264.7 macrophage cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in plastic culture flasks in DMEM containing

L-glutamine supplemented with 10% foetal calf serum (FCS) and 1% PSF (penicillin/streptomycin/fungizone) solution under 5% CO<sub>2</sub> at 37 °C. The solution was split bi-weekly and all cells were seeded in 96-well microtitre plates. The cells were then activated by incubation in a medium containing LPS (1 µg/ml) and various concentrations (100, 50, 25, and 12.5 µg/ml) of the methanol plant extracts dissolved in DMSO with a final DMSO concentration of 0.2%.

### 5.2.2.2 Quantification of NO Release

The concentration of NO in culture medium was evaluated using the Griess reagent assay (HUNTER et al., 2013). A 96-well plate was used to seed the RAW 264.7 cells at 10<sup>5</sup> cells/well. Seeding the wells at 10<sup>5</sup> or 100 000 cells per well is done by quantifying the number of cells in the cell culture medium first. An aliquot of cells is counted using a haemocytometer which enables the calculation of the number of cells/ml. Then the cell suspension was diluted to the required concentration using fresh medium before pipetting the known number of cells into the wells at a density of 4 X 10<sup>4</sup> cell/ml.

The cells were washed with a buffer solution, Phosphate Buffer Solution (PBS), frequently used in research of a biological nature, a water-based salt solution containing disodium hydrogen phosphate 1.42 g/l, sodium chloride 8 g/l, potassium chloride 0.2 g/l and potassium dihydrogen phosphate 0.24 g/l. The osmolality and ion concentrations of the solutions match those of the human body (isotonic). The pH was adjusted to 7.4 with HCl and distilled water added to form a total volume of 1 litre. The resultant 1x PBS had a final concentration of 10 mM PO<sub>4</sub><sup>3-</sup>, 137 mM NaCl, and 2.7 mM KCl. If used in cell culturing, the solution can be dispensed into aliquots and sterilized by autoclaving or filtration. PBS was stored in the refrigerator until further use. The concentrated stock solutions may become precipitous upon cooling and were thus maintained at room temperature until the precipitate had completely dissolved prior to use.

The cells were then pre-treated with several concentrations of plant extracts and 1 µg/ml of LPS for 24 h. When pre-treating the cells, defined concentrations of plant extract (100, 50, 25 and 12, 5 µg/ml) together with LPS stimulates NO production in the medium in which the cells are incubated. The supernatant (100 µl) was combined with the equivalent volume of Griess reagent and placed into an incubator for 15 min in the dark. The absorbance of the water-soluble purplish-red product was read on a BioTek Synergy microplate reader after 10 min at 550 nm. The amount of NO was calculated by a calibration curve established with 0.15-100 µM NaNO<sub>2</sub>.

The inhibition percentage was calculated based on the ability of the extracts to inhibit NO formation by cells compared with the control (cells in media without compounds containing triggering agents and DMSO), which was considered as 0% inhibition. A graph of percentage inhibition against the concentration of the different compounds were used to calculate the IC<sub>50</sub> values.

### 5.2.2.3 Cytotoxicity Assay

The cytotoxicity assay used in the study followed a method described by **MOSMANN (1983)** and **McGAW et al. (2000)**, with slight modifications as indicated in Chapter Four in Section 4.2.2.

### 5.2.3 Lipoygenase (LOX) Inhibition Assay

This evaluation was conducted using the guidelines of a procedure described by **PINTO et al. (2007)** with minor modifications. The assessments were based on measuring the production of the composite Fe<sup>3+</sup>/xylenol orange using a spectrophotometer at 560 nm. The enzyme 15-lipoxygenase from *Glycine max* was incubated with plant extracts or a standard inhibitor at 25 °C for 5 min. Then linoleic acid (final concentration 140 µM) in Tris-HCl buffer (50 mM, pH 7.4) was added and the mixture was incubated at 25 °C for 20 min in the dark. The reaction was terminated by the addition of 100 µL of FOX reagent consisting of sulphuric acid (30 mM), xylenol orange (100 µM), iron (II) sulphate (100 µM) in methanol/water (9:1; v/v). For the control, only LOX solution and buffer were pipetted into the wells. The enzyme LOX was present in the blanks during incubation and the substrate (linoleic acid) was added after the FOX reagent. The inhibitory activity of lipoxygenase was determined by calculating the inhibition percentage of hydrogen peroxide produced from the modifications in absorbance values at 560 nm after 30 min at 25 °C. Inhibition percentage was calculated as follows:

$$\text{Percentage inhibition} = ((A_{\text{control}} - A_{\text{blank}}) - (A_{\text{sample}} - A_{\text{blank}})) / (A_{\text{control}} - A_{\text{blank}}) \times 100$$

Where, A<sub>control</sub> is the absorbance of control well, A<sub>blank</sub> is the absorbance of blank well and A<sub>sample</sub> is the absorbance of sample well.

## 5.3 Results and Discussion

### 5.3.1 Inhibition of NO activity

The calculated concentration of an inhibitor known as the IC<sub>50</sub> is known to be determined where the response (or binding) is condensed by half. It applies to the inhibition of targets like an

isolated enzyme and is a quantity of the efficiency of a substance that is required to inhibit a specific biochemical or biological function. The values are typically expressed as micrograms per millilitre. It is often used as a measure of adverse drug potency in ethnopharmacological research. Potency is a measure of drug activity expressed in terms of the amount required to produce an effect of given intensity. A highly potent drug induces a given response at low concentrations, while a drug of lower potency evokes the same response only at higher concentrations. The potency depends on both the affinity and efficacy. According to the FDA,  $IC_{50}$  represents the concentration of a drug that is required for 50% inhibition *in vitro* (ADEBAYO et al., 2013; ADEBAYO et al., 2015).

Values of the  $IC_{50}$  for the flavonoid control, quercetin was 6.30  $\mu\text{g/ml}$  (Table 5.1). All plant extract values were considered in relation to the control. Values greater than 100  $\mu\text{g/ml}$  indicated less active anti-inflammatory agents and plant extracts that fell in this category from this study were the leaf extracts of the three *Aloe* species (*A. arborescens*, *A. aristata*, and *A. ferox*) and *H. limifolia*. There seem to be a marked difference between the leaf and the stem extracts of *T. riparia* in terms of the inhibition potential of NO with the stem demonstrating an almost seven-fold efficacy to that of the leaf extract at an  $IC_{50}$  value of 13.99  $\mu\text{g/ml}$ . Further significant  $IC_{50}$  values were observed for *H. aethiopicum* leaf extract (22.47  $\mu\text{g/ml}$ ) and *M. plumbea* extract for the leaf at 29.35  $\mu\text{g/ml}$ , the bulb at 22.04  $\mu\text{g/ml}$  and the root extract at 26.93  $\mu\text{g/ml}$ ). In addition, the *Bulbine* species were observed to have an  $IC_{50}$  value for *B. natalensis* root extract at 28.64  $\mu\text{g/ml}$  and *B. frutescens* leaf extract at 26.32  $\mu\text{g/ml}$  were also significant showing active anti-inflammatory characteristics towards NO inhibition.



**Table 5.1** IC<sub>50</sub> (µg/ml) of Nitric oxide of different medicinal plant extracts used for skin disorders and wound healing

<b>Plant Extracts</b>	<b>IC<sub>50</sub> (µg/ml)</b>
<i>Aloe arborescens</i> leaf	> 100
<i>Aloe aristata</i> leaf	> 100
<i>Aloe ferox</i> leaf	> 100
<i>Bulbine frutescens</i> leaf	<b>*26.32 ± 0.69</b>
<i>Bulbine frutescens</i> root	42.51 ± 1,69
<i>Bulbine natalensis</i> leaf	54.79 ± 13,47
<i>Bulbine natalensis</i> root	<b>*28.64 ± 4.58</b>
<i>Eucomis autumnalis</i> leaf	38.09 ± 2.21
<i>Eucomis autumnalis</i> bulb/root	63.10 ± 6.59
<i>Haworthia limifolia</i> leaf	> 100
<i>Hypericum aethiopicum</i> leaf	<b>*22.47 ± 3.87</b>
<i>Merwillia plumbea</i> leaf	<b>*29.35 ± 0.55</b>
<i>Merwillia plumbea</i> bulb	<b>*22.04 ± 0.20</b>
<i>Merwillia plumbea</i> root	<b>*26.93 ± 2.60</b>
<i>Tetradenia riparia</i> leaf	<b>*13.99 ± 2.47</b>
<i>Tetradenia riparia</i> stem	2.05 ± 1.18
<i>Zantedeschia aethiopica</i> leaf	57.94 ± 2.22
<i>Zantedeschia aethiopica</i> stem	46.22 ± 5.52
Quercetin	6.30 ± 0.41

\*Bold indicates the plant extracts with active anti-inflammatory activity.

The inflammation process is a highly complex pathophysiological process which occurs when various processes of various molecules and mediators that are signalling, occur in a series of catalytic enzymatic reactions (**WHITE, 1999**). Many plant extracts are known to exert their inhibitory enzyme effects via a range of diverse action mechanisms and target sites (**CAPONE et al., 2007**). Many studies with various plant species have reported good inhibitory activity (**JÄGER et al., 1996; TAYLOR and VAN STADEN, 2001; JÄGER and VAN STADEN, 2005; FAWOLE et al., 2009**). Superoxide and nitric oxide (NO) production are associated with the development of several diseases. Recently it has been shown that interactions between them may also be important in disease pathology. The central hypothesis emerging is that the balance between nitric oxide and superoxide generation is a critical determinant in the etiology

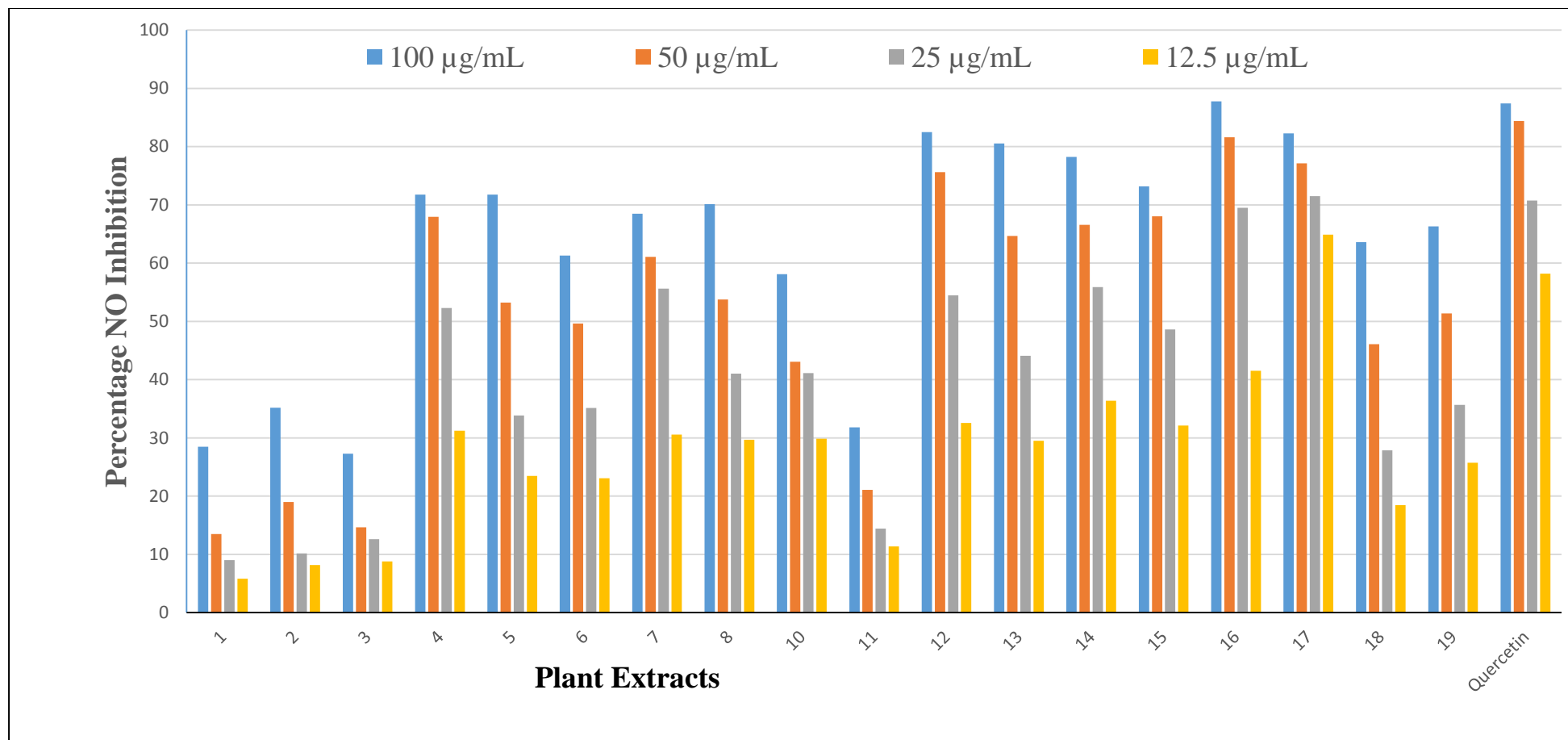
of many human diseases including atherosclerosis, neurodegenerative disease, ischemia-reperfusion and cancer. These are important in the context of the current and future status of therapies which could modulate the balance between nitric oxide and superoxide. Nitric oxide and reactive oxygen species exert multiple modulating effects on inflammation and play a key role in the regulation of immune responses. They affect virtually every step of the development of inflammation. Low concentrations of nitric oxide produced by constitutive and neuronal nitric oxide synthases inhibit adhesion molecule expression, cytokine and chemokine synthesis and leukocyte adhesion and transmigration. Large amounts of NO, generated primarily by iNOS can be toxic and pro-inflammatory. Actions of nitric oxide are, however, not dependent primarily on the enzymatic source, but rather on the cellular context, NO concentration (dependent on the distance from NO source) and initial priming of immune cells (**DARLEY-USMAR, WISEMAN, HALLIWELL, 1995; GUZIK, KORBUT, ADAMEK-GUZIK, 2003**). The results of this study show that the enzyme inhibitory activity of stem, bulb, leaf and root extracts of the different plant parts were significant in their anti-inflammatory capacity.

In Figure 5.2 below, the percentage of NO inhibition is represented for the four different concentrations (12.5, 25, 50 and 100  $\mu\text{g/ml}$ ) at which each of the plant extracts were tested. A dose-dependent trend was observed for all the plant extracts. Low levels of inhibition were observed for all concentrations of the three *Aloe* species leaves and the *H. limifolia* leaf. This can be explained as low percentage NO inhibition activity for these plant extracts. The high percentage inhibition (at 100  $\mu\text{g/ml}$ ) was observed for many of the other plant extracts and ranged from above 50% to just below 90% comparable to that of the quercetin control which yielded around 85-88% NO inhibition. The medicinal plant extracts for *M. plumbea*, *T. riparia*, *Z. aethiopica*, *H. aethiopicum* and *E. autumnalis* were significant for all concentrations. Unstimulated macrophages in culture medium produce background levels of nitrite in the culture medium of around 0.09  $\mu\text{g/ml}$ . The treated cells with different concentrations of extracts have significant inhibition of nitrite production. Although the results in Figure 5.2 are of less significance in this context as they were used to calculate the  $\text{IC}_{50}$  values in Table 5.1. The  $\text{IC}_{50}$  values represent the effective concentration of the extracts that inhibit 50% of the NO.

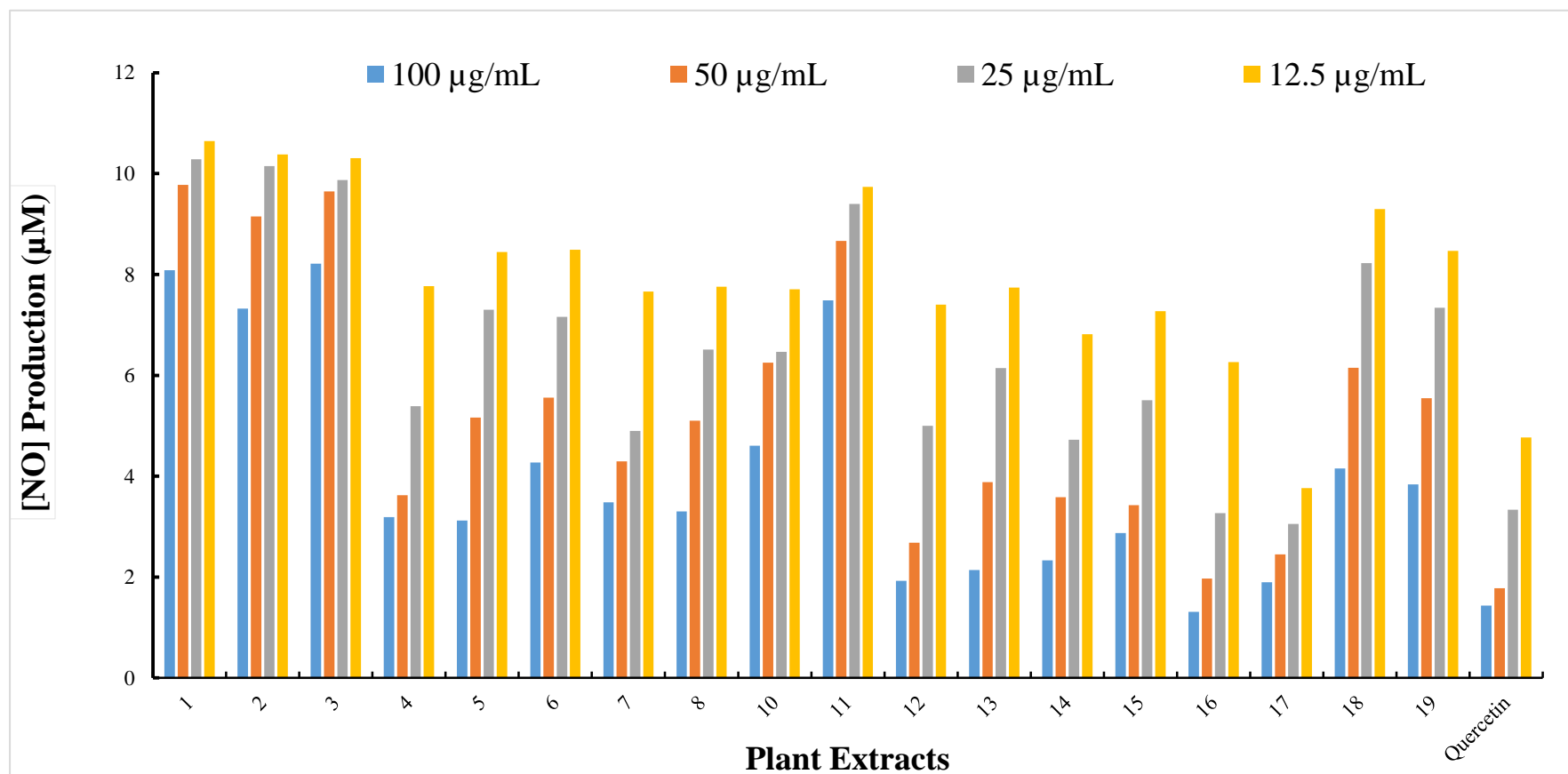
Figure 5.3 below represents the  $\mu\text{M}$  NO production at various concentrations (12.5, 25, 50 to 100  $\mu\text{g/ml}$ ) of the plant extracts. The quercetin positive control values of NO production ( $\mu\text{M}$ ) showed a pattern or trend. At the lower concentrations of plant extracts the cell viability values were higher. This pattern was seen for all the plant extracts. The leaves of the three *Aloe* species, *H. limifolia* leaf and *H. aethiopicum* and *Z. aethiopica* leaf were observed to have high production of NO. At the higher concentration of plant extracts, NO production was lower.

What does this mean for medicinal use in skin disorders and wound healing? **DZOYEM and ELOFF (2015)** reported that cytotoxicity and inflammation occur when over-production of NO occurs in the body. NO production is important as an anti-inflammatory agent. Quercetin as a positive control, significantly reduces NO production in the LPS-stimulated RAW 264.7 murine macrophage cell lines (**DZOYEM and ELOFF, 2015**).

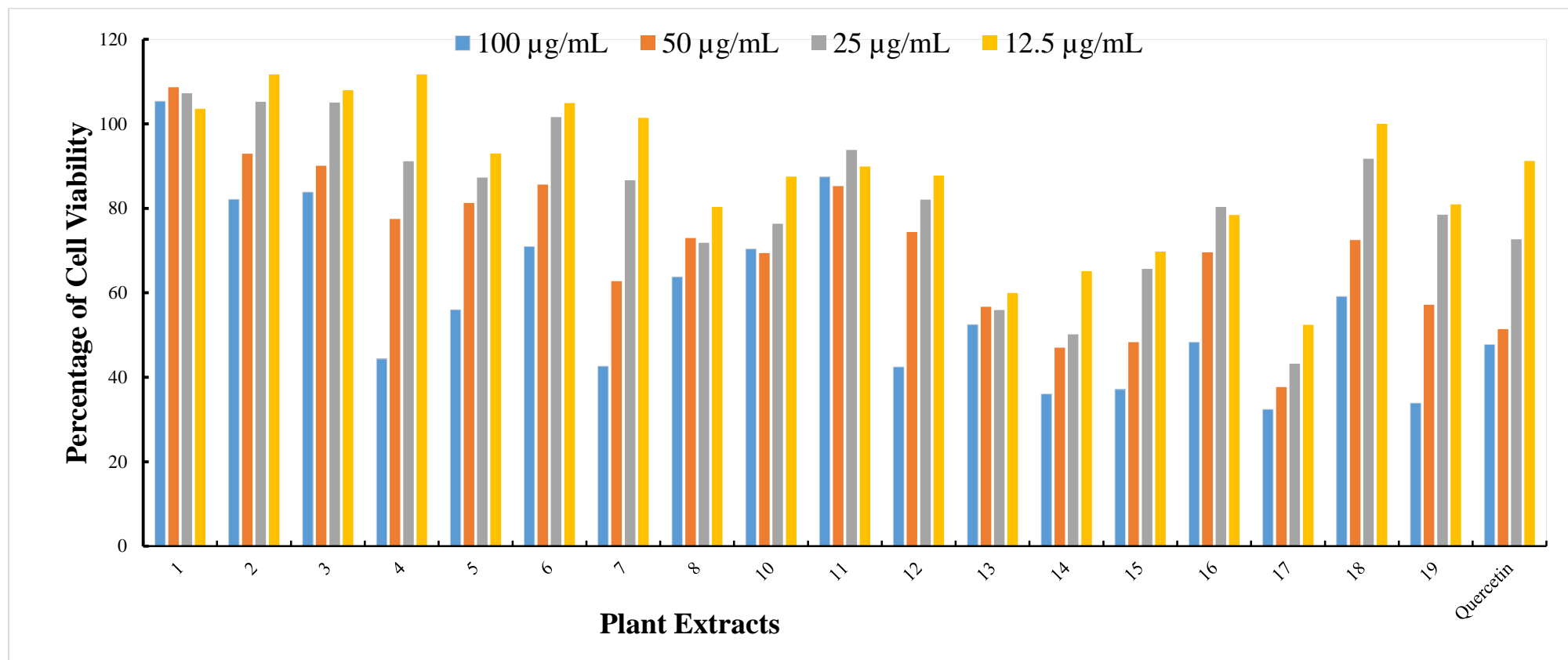
Figure 5.4 below are the outcomes of the MTT assay used to determine the percentage of cell viability for the different cell extracts. NO inhibition of the plant extracts was associated with their cytotoxicity on RAW 264.7 macrophages. A percentage of the control cells compared to viable cells that were incubated in fresh culture medium. The results of cell viability levels were high for most of the extracts at the lower concentration. The capacity for medicinal plants to be used in the management and the healing process during wounds and skin diseases may be supported by this assay. Its importance as tool in identifying the effects of the various plant extract concentrations on the viability and/or survival of mammalian cells was observed. The plant extract activity which is inhibitory on NO production by induced RAW 264.7 macrophage cell lines indicated good activity for low cytotoxicity and production of NO for plants used in wound healing and skin conditions (**ADEBAYO et al., 2013; 2015**). The release of NO promotes inflammation, and so the extracts act as foragers of NO and production inhibitors, together with low cytotoxicity which is indicative of mitigating the proliferation of inflammation by NO. The NO production inhibition in medicinal plants may be due to inducible nitric oxide synthase activity expression. The effects of both nitric oxide and superoxide in immune regulation are exerted through multiple mechanisms, which include interaction with cell signaling systems like cGMP, cAMP, G-protein, JAK/STAT or MAPK dependent signal transduction pathways. They may also lead to modification of transcription factors activity and in this way modulate the expression of multiple other mediators of inflammation (**DARLEY-USMAR, WISEMAN, HALLIWELL, 1995; GUZIK, KORBUT, ADAMEK-GUZIK, 2003; LEE et al., 2003; 2007; 2010**).



**Figure 5.2** Percentage of NO inhibition at different concentrations of plant extracts. 1 - *Aloe arborescens* leaf, 2 - *Aloe aristata* leaf, 3 - *Aloe ferox* leaf, 4 - *Bulbine frutescens* leaf, 5 - *Bulbine frutescens* root, 6 - *Bulbine natalensis* leaf, 7 - *Bulbine natalensis* root, 8 - *Eucomis autumnalis* leaf, 10 - *Eucomis autumnalis* bulb/root, 11 - *Haworthia limifolia* leaf, 12 - *Hypericum aethiopicum* leaf, 13 - *Merwillia plumbea* leaf, 14 - *Merwillia plumbea* bulb, 15 - *Merwillia plumbea* root, 16 - *Tetradenia riparia* leaf, 17 - *Tetradenia riparia* stem, 18 - *Zantedeschia aethiopica* leaf, 19 - *Zantedeschia aethiopica* stem.



**Figure 5.3** Concentration of NO Production in  $\mu\text{M}$  for the different plant extracts. 1 - *Aloe arborescens* leaf, 2 - *Aloe aristata* leaf, 3 - *Aloe ferox* leaf, 4 - *Bulbine frutescens* leaf, 5 - *Bulbine frutescens* root, 6 - *Bulbine natalensis* leaf, 7 - *Bulbine natalensis* root, 8 - *Eucomis autumnalis* leaf, 10 - *Eucomis autumnalis* bulb/root, 11 - *Haworthia limifolia* leaf, 12 - *Hypericum aethiopicum* leaf, 13 - *Merwillia plumbea* leaf, 14 - *Merwillia plumbea* bulb, 15 - *Merwillia plumbea* root, 16 - *Tetradenia riparia* leaf, 17 - *Tetradenia riparia* stem, 18 - *Zantedeschia aethiopica* leaf, 19 - *Zantedeschia aethiopica* stem.



**Figure 5.4** Percentages of cell viability with MTT assay at different concentrations of plant extracts. 1 - *Aloe arborescens* leaf, 2 - *Aloe aristata* leaf, 3 - *Aloe ferox* leaf, 4 - *Bulbine frutescens* leaf, 5 - *Bulbine frutescens* root, 6 - *Bulbine natalensis* leaf, 7 - *Bulbine natalensis* root, 8 - *Eucomis autumnalis* leaf, 10 - *Eucomis autumnalis* bulb/root, 11- *Haworthia limifolia* leaf, 12 - *Hypericum aethiopicum* leaf, 13 - *Merwillia plumbea* leaf, 14 - *Merwillia plumbea* bulb, 15 - *Merwillia plumbea* root, 16 - *Tetradenia riparia* leaf, 17 - *Tetradenia riparia* stem, 18 - *Zantedeschia aethiopica* leaf, 19 - *Zantedeschia aethiopica* stem.

### 5.3.2 LOX Inhibitory Activity

One of the important objectives of this study was to determine the activity of the selected extracts for anti-inflammatory capacity using a LOX model of inhibition. Table 5.2 provides details of the IC<sub>50</sub> (µg/ml) values using the quercetin control inhibition of 15.91 µg/ml against which all plant extracts were compared. All the plant extracts in the study, except the three *Aloe* species extracts, the two *Bulbine* species extracts, as well as extracts of *H. limifolia*, *M. plumbea* and *Z. aethiopica* showed significant biological activity at low concentrations. *T. riparia* plant part extracts ranged from 3.55 to 8.50 µg/ml, *H. aethiopicum* leaf extract at 4.45 µg/ml and *E. autumnalis* plant extracts from 6.73 to 9.52 µg/ml and *Z. aethiopica* extract at 9.05 µg/ml for the LOX inhibitory activity assay. These results indicate significantly positive anti-inflammatory activity. Values greater than 100 µg/ml indicate less activity. Those lower than the standard control values are *T. riparia* (leaf extract - 8.50 µg/ml, stem extract - 3.55 µg/ml), *H. aethiopicum* (4.45 µg/ml), *E. autumnalis* (leaf extract - 6.73 µg/ml, bulb/root extract - 9.52 µg/ml), *B. natalensis* leaf extract (6.93 µg/ml) and *Z. aethiopica* stem extract (9.05 µg/ml).

A study on *E. autumnalis* leaves detected between 80-90% which is high activity for COX-1 inhibition *in vitro* with plant lectins contributing to the anti-inflammatory properties (GAIDAMASHVILLI and VAN STADEN, 2006). FAWOLE et al. (2009; 2010), reported *A. ferox* leaves as having the highest COX-2 anti-inflammatory activity. The LOX group of enzymes plays an important role in many inflammatory disorders (CHEDEA and JISAKA, 2005; SCHEINDER and BUCAR, 2005). *Hypericum* species have been reported by various researchers as having positive antimicrobial activity (SIDDIQUE et al., 2010), the presence of phenolic compounds and terpenoids and the *in vivo* wound healing activity in rats (MUKHERJEE et al., 2003) were comparable to the effects of a standard allopathic drug called nitrofurazone ointment. *Z. aethiopica* is known for its sterols and triterpenoids which may attribute to its anti-inflammatory activity (VAN WYK et al., 2009). *T. riparia* is one of the maximally utilized and widespread medicinal plants in Rwanda and various new bioactive substances were isolated from its leaves including a diterpene diol with high antimicrobial activity (VAN PUYVELDE et al., 1986). In *M. plumbea* extracts, the flavonoid content may be the reason for its anti-inflammatory properties and usage by traditional healers (DYSON et al., 1998; VAN WYK et al., 2009). Researchers in a South African study have reported *H. limifolia* as having good antimicrobial activity (COOPOOSAMY and NAIDOO, 2011a). Another study on *E. autumnalis* by MASONDO et al. (2014), reports ethnopharmacological

potential including anti-inflammatory activity. **VAN WYK et al. (2009)** reported the presence of lectin-like proteins with anti-inflammatory activity in *Eucomis* species, with the bulb having significant COX-2 inhibitory action (**TAYLOR and VAN STADEN, 2001**) and triterpenoids beneficial in wound healing therapy (**REYNEKE, 1980**). The need for conservation of these plants is critical as trade and demand increase (**MANDER, 1997; DOLD and COCKS, 2002**). *Bulbine* species, *E. autumnalis*, *H. limifolia* are some of the over-harvested medicinal plants in the Eastern Cape while in KwaZulu-Natal *H. aethiopicum*, *E. autumnalis*, *M. plumbea* and *H. limifolia* are in great demand. *Eucomis* species in Limpopo Province is also one of eight most frequently traded medicinal plants (**MOENG and POTGIETER, 2011**). According to a recent report on a wide range of South African plants by **WILLIAMS et al. (2013)**, the usage of medicinal plants that are endangered and threatened include all species of *Haworthia*, *Aloe*, *Eucomis* and *Tetradenia*. It is due to demands for medicinal use, marketability and export that lead to extensive harvesting pressures in the field to the extent that some of these plants can be reduced to possible total extinction and are currently on the red data list in South Africa. The education of the people towards effective conservation is essential for sustainability **WILLIAMS et al. (2013)**.

This study may contribute to overcoming the conservation concerns for these species. The ethnopharmaceutical activities of the various plant parts tested can be used for conservation information through evidence-based systems. Plant protection, promotion and education with scientific evidence is essential for sustainability and the conservation of red data listed plants in South Africa.



**Table 5.2** IC<sub>50</sub> (µg/ml) levels detected for Lipoxygenase in the different plant extracts

Plant Extracts	IC <sub>50</sub> (µg/mL)
<i>Aloe arborescens</i> leaves	> 100
<i>Aloe aristata</i> leaves	> 100
<i>Aloe ferox</i> leaves	> 100
<i>Bulbine frutescens</i> leaves	>100
<i>Bulbine frutescens</i> roots	81.54 ± 6.76
<i>Bulbine natalensis</i> leaves	<b>*6.93 ± 0.28</b>
<i>Bulbine natalensis</i> roots	98.54 ± 2.10
<i>Eucomis autumnalis</i> leaves	<b>*6.73 ± 0.11</b>
<i>Eucomis autumnalis</i> bulbs/roots	<b>*9.52 ± 0.11</b>
<i>Haworthia limifolia</i> leaves	> 100
<i>Hypericum aethiopicum</i> leaves	<b>*4.45 ± 0.23</b>
<i>Merwillia plumbea</i> leaves	32.57 ± 0.37
<i>Merwillia plumbea</i> bulbs	64.95 ± 3.40
<i>Merwillia plumbea</i> roots	>100
<i>Tetradenia riparia</i> leaves	<b>*8.50 ± 0.44</b>
<i>Tetradenia riparia</i> stems	<b>*3.55 ± 0.11</b>
<i>Zantedeschia aethiopica</i> leaves	>100
<i>Zantedeschia aethiopica</i> stems	<b>*9.05 ± 1.61</b>
Quercetin	15.91 ± 3.02

\*Bold indicates significant plant extracts with lipoxygenase content.

The LOX enzyme is considered to produce prostaglandins in acute inflammation (**VANE and BOTTING, 1987; VANE and BOTTING, 1995; VANE et al., 1998**). Research showed that in some muscles and tissues such as the reproductive organs of the female, brain, placenta, kidneys, the enzyme is manufactured at a consistent rate, and synthesises prostanoids are accountable for regulation and homeostasis in these muscles and tissues (**HINZ and BRUNE, 2002; MITCHELL and WARNER, 2006**). Selective inhibition of some anti-inflammatory compounds are the most favourable because they diminish the detrimental side effects associated with inhibition.

The medicinal plants investigated in many instances showed good LOX and NO inhibition activity. Those with moderate activity are preferable to use rather than the ones with high

activity. Prolonged use of plant extracts with high inhibition activity causes the expression of damaging symptomatic side effects. The plant extracts that were significantly positive for both NO and LOX screening for anti-inflammatory activity in relation to quercetin include *T. riparia*, *E. autumnalis*, *H. aethiopicum* and *H. limifolia*. The IC<sub>50</sub> values for the NO and the LOX inhibition in plants like *T. riparia* and *H. aethiopicum* in particular had significant outcomes in both assays. *T. riparia* and *H. aethiopicum* are the two plant species which also showed consistent skin and wound healing properties.

## 5.4 Conclusions

Developing safe and effective anti-inflammatory agents from medicinal plants showing LOX and COX-2 inhibition are acknowledged as safer medicines with minimum side effects *in vivo*. Some of the plants screened in this study exhibited some good anti-inflammatory activity, however, further research is needed to isolate the bioactive compound/s. This may provide valuable leads towards the discovery of agents that have anti-inflammatory abilities. The plants screened in this study are utilized by traditional healers as a management tool or natural resource for healing wounds and for skin disorders. The mode of action for the dermatological use of medicinal plants is sometimes determined by their phenolic content, positive anti-inflammatory, antioxidant activity and protein interaction properties. These are complex yet intricate pathways, each contributing a part to the whole or bigger picture in the important role medicinal plants have to play in traditional health care. Medicinal plants may not display activity at one pharmacological site but may act at several potential targets, hence the various biological assays assist with proper evaluation of the mode of action of the selected medicinal plants.

## CHAPTER SIX

### ANTIOXIDANT ACTIVITY AND WOUND HEALING PROPERTIES OF PLANT EXTRACTS

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#### 6.1 Introduction

Antioxidants are substances or compounds which, at reduced concentrations slow down or prevent oxidation, counteracting free radicals thereby preventing oxidative damage (**BECKER et al., 2004**). They are known to occur in various medicinal plants and are usually related to the presence of phenolics which are the most abundant class of antioxidants (**SCALBERT et al., 2005**). Polyphenols such as tannins and flavonoids, tocopherols and catechins are amongst the documented antioxidants (**SCALBERT et al., 2005**). Organic acids, carotenoids and protein hydrolysates also exhibit antioxidant or synergistic effects with other antioxidants (**DAPKEVICIUS et al., 1998**). Free radicals are linked to the inflammation processes; so when the immune system is compromised they are found to recruit inflammatory cells (**ALLEN, 2003; AYYAGARRI et al., 2003**). Several biochemical reactions are known to provide free radicals to the body and these are associated with many diseases, increased ageing and poor healing of wounds and injuries to the skin. The resulting consequences are due to unregulated production of oxygen free radicals and an unstable mechanism of antioxidant security (**TSAO and DENG, 2004; AL-DABBAS, 2006; AKULA and ODHAV, 2008; HAYET et al., 2008**). While in other studies when oxidants are present at low concentrations compared to that of oxidizable substrates, delays or inhibition of oxidation of the substrates occur (**PERCIVAL, 1998; YOUNG and WOODSIDE, 2001; ATOUI et al., 2005**). Free radical and hydroxyl free radical scavengers' physiological role is to prevent damage to cellular components due to chemical reactions. Most importantly, free radicals contribute to the physiological processes of ageing, disease progression, degeneration diseases and prolonged lifespan (**AMES et al., 1990; PERCIVAL, 1998; YOUNG and WOODSIDE, 2001; GUTTERIDGE and HALLIWELL, 2010**). Antioxidants are known to naturally control free radical formation (**PERCIVAL, 1998**). When the availability of antioxidants is reduced the damage increases and stressful oxidation occurs. Antioxidants are recognized as having the ability to stabilize and deactivate free radicals before they cause damage to cells and biological structures and are critical in overall well-being and for the maintenance of optimum cellular and general systemic health (**PERCIVAL, 1998**). Thus the role of free radicals has to be balanced and may

contribute to both pro- and contra-indicatory consequences in the human body. **LOBO et al. (2010)** reported an extensive study on free radical chemistry. These free radicals are reactive oxygen and nitrogen species that are generated by the body through multiple endogenous systems, which are the result of exposure to various physiochemical conditions. A form of effective balance between free radicals and antioxidants are important for the mammalian body to function physiologically normal (**LOBO et al., 2010**). Should circumstances arise for free radicals to overwhelm the body's ability to regulate them, then an oxidative stress situation will arise. The free radicals will negatively alter the bodies' lipids, proteins and DNA thereby triggering various human diseases. Synthetic antioxidants like butylated hydroxytolulene and butylated hydroxyanisole are reported to be harmful to human health. Hence there is a need for research for effective, nontoxic natural compounds with antioxidative activity (**LOBO et al., 2010**).

According to **KURAHASHI and FUJII, 2015**; oxygen plays a fundamental role in the wound healing process. These functions include oxidative bacterial killing, collagen synthesis, angiogenesis and epithelialization. The wound healing process is impaired under hypoxia, however the exact role of oxygen in wound healing is not understood as yet. Although oxygen is used to produce energy via oxidative phosphorylation, reactive oxygen species (ROS) are produced and cause oxidative injury. The cells of aerobic organisms constantly produce ROS during normal metabolic processes and their production is elevated under pathological conditions. Although ROS supports various physiological processes, they also impact on life-threatening damage. In the wound healing process, the physiological role and the molecular mechanisms of ROS-involved reactions have been investigated for individual aspects. In particular, the pathological role of ROS in the inflammatory phase is known. In the inflammatory phase, neutrophils and macrophages arrive at a wound lesion and start to secrete large amounts of ROS along with pro-inflammatory cytokines and proteolytic enzymes such as matrix metalloproteinase (MMP). NADPH oxidase (NOX<sub>2</sub>) is expressed at high levels in plasma membranes of inflammatory cells and activated during phagocytosis, which results in the production of large amounts of superoxide radical anions. The generated ROS directly attack invading pathogens, and finally kill them to aid phagocytosis. Excessively produced superoxide is known to damage the surrounding tissues. Superoxide is dismutated to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and molecular oxygen by either superoxide dismutase (SOD) or a spontaneous reaction (**KURAHASHI and FUJII, 2015**). H<sub>2</sub>O<sub>2</sub> is detoxified by peroxidases such as catalase, glutathione peroxidase (GPX), and peroxiredoxin (PRDX) to avoid the Fenton reaction that occurs in the presence of transition metal ions such as iron or copper and generates

hydroxyl radicals, the most harmful ROS. Compared to immune cells, ROS are produced by other cells at much lower levels. Low levels of ROS play a physiological role, notably as cellular signaling in response to stimuli. The role of ROS signaling in angiogenesis has been well studied. Moderate levels of H<sub>2</sub>O<sub>2</sub> up-regulate the production of the vascular endothelial growth factor (VEGF), the key angiogenic growth factor, in keratinocytes, and result in accelerating angiogenesis. Both NO<sub>2</sub> and NO<sub>4</sub> play pivotal roles in modulating vascular endothelial cell proliferation, but they function differentially in vasculogenesis via ROS generation. ROS are also involved in re-epithelialization. Excessive ROS have decelerating effects on angiogenesis. In some enzymes involved in signaling pathways, such as phosphotyrosine phosphatase, sulfhydryl residues that act as the catalytic center are highly sensitive to oxidative modification, and tend to suffer from oxidative inactivation (**KURAHASHI and FUJII, 2015**). Thus, excess ROS strain signaling networks through creating an imbalanced redox homeostasis, resulting in impaired wound healing. Diabetes, aging, immunodeficiency, and malnutrition are typical causes for delayed wound healing. Under these pathological conditions, redox imbalance occurs, and elevated oxidative injury is observed (**KURAHASHI and FUJII, 2015**).

Research shows that many isolated antioxidants from plants are polyphenols, with additional biological activities that range from anti-inflammatory, antimicrobial, anti-carcinogenic, anti-allergic, immune-stimulatory and oestrogenic activity (**LARSON, 1988**). Antioxidant activity of phenolic compounds is mainly due to their redox properties allowing them to function as reducing agents, hydrogen donors and singlet oxygen quenchers; as well as metal chelators. Some food products are known to contain synthetic antioxidants like tertbutyl-hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) however concerns about the safety of synthetics have led to the progress of research in natural antioxidants (**LARSON, 1988**). Polyphenols are known for a wide variety of other related beneficial effects such as lipoxygenase inhibitory properties and the treatment of anti-inflammatory diseases (**SREEJAYAN and RAO, 1996**).

Antioxidant assays have been routinely used for many years (**NDHLALA et al., 2010; NDHLALA et al., 2014; NDHLALA et al., 2015**) and provide reliable outcomes on the activity of medicinal plants. In this study, three *in vitro* methods, the electron transfer based assays namely the ferric reducing antioxidant power (FRAP), the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and a hydrogen transfer based assay;  $\beta$ -

carotene linoleic acid bleaching assay (CLAMS) were used to screen the medicinal plants investigated.

The DPPH antioxidant assay involves the use of a stable free radical that reacts well with other free radicals which are unstable and are more reactive. The DPPH antioxidant method was developed from the mechanism of searching of DPPH by antioxidants resulting in a reduction reaction causing decolourization of deep purple DPPH methanol solutions. In the DPPH assay intensity of yellow colour depends on the amount and nature of radical scavenger present in the samples. The method measures the reducing capability of the DPPH antioxidants radical using a spectrophotometric reading. This is a simple, commonly used method with accurate results and quantifies extracts with hydrophilic or lipophilic antioxidants (NDHLALA et al., 2010; NDHLALA et al., 2013).

The FRAP method is a simple, fast and robust method based on the redox reaction of compounds and quantifies extracts with hydrophilic and lipophilic antioxidants (NDHLALA et al., 2010). One limitation of this method is that a compound with a redox potential less than 0.70 Volts can decrease iron despite not performing as an antioxidant *in vivo* (PEREZ-JIMENEZ et al., 2008). Some antioxidants such as proteins and thiols are not measured by the FRAP assay, however, the method is accomplished at a non-physiological pH and the ferric ion may then be irrelevant physiologically (PEREZ-JIMENEZ et al., 2008; NDHLALA et al., 2010; NDHLALA et al., 2013).

The  $\beta$ -carotene linoleic acid bleaching (CLAMS) assay measures the inhibition capacity of volatile organic production and the formation of combined diene hydro peroxidase due to linoleic acid oxidation which lightens the  $\beta$ -carotene in the extracts. Temperature regulation which is critical for induced oxidation and discolouration is inhibited by the antioxidants that donate hydrogen atoms to supply radicals (NDHLALA et al., 2010).

### 6.1.1 The Role of Phenols as Antioxidants

Research showing the beneficial roles of phenolic compounds as natural antioxidants have identified and/or led to the isolation of potent simple and polymeric phenolic compounds (BIEWENGA et al., 1997; NENADIS et al., 2003; ATOUI et al., 2005; DE ALMEIDA-MELO et al., 2005; SOUZA et al., 2007; KUMAR and NAIK, 2010; MEGHASHRI et al., 2010). Epidemiological studies have reported phenolic compounds as natural antioxidants that neutralise free radicals and function as metal chelators (KIM et al., 1997; VELIOGLU et al.,

1998; MUCHUWETI et al., 2006; CHITINDINGU et al., 2007; KHALIL et al., 2007a; 2007b; NDHLALA et al., 2007; NDHLALA et al., 2008; ALI et al., 2008; ANDUEZA et al., 2009; KIM et al., 2009; MOYO et al., 2010). Hydroxyl groups in the chemical structure of polyphenols make them suitable for free radical scavenging reactions and as metal chelating agents (LOPEZ et al., 2003). The organization of the hydroxyl groups around the phenolic molecule is important for antioxidant reactions (MOURE et al., 2001). HAGERMAN et al. (1998) reported that the phenolic acid antioxidant potential improves as the number of methoxyl and hydroxyl groups increases and greater antioxidant ability of condensed and hydrolysable tannins is observed. Phenolic compounds are a major group of compounds that are abundant in plants (NDLALA et al., 2010).

The study objectives were to determine and assess the antioxidant properties of the selected medicinal plant extracts frequently used for wound healing and skin disorders using three *in vitro* model systems (DPPH, FRAP, CLAMS) as well as protein precipitation properties.

## 6.2 Materials and Methods

### 6.2.1 Collection and Preparation of Plant Material

Plant material extracts were prepared as indicated in Chapter 2, Sections 2.4.1 and 2.4.2.

### 6.2.2 Antioxidant Activity of Plant Extracts

#### 6.2.2.1 2, 2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity

The DPPH free radical scavenging activity of the 19 plant extracts were determined using the assay as described by KARIOTI et al. (2004). Dried plants extracts were redissolved to 10 mg/ml in 50% (v/v) aqueous methanol. Various concentrations (1, 0.5, 0.25, 0.125, 0.0625, 0.031, 0.015, 0.0008 mg/ml) of the diluent plant extract were tested. Fifteen microliters of each plant extract were diluted in a test tube with 735  $\mu$ l of methanol and 750  $\mu$ l of 0.1 mM DPPH (Sigma-Aldrich, Germany) of freshly prepared methanolic DPPH solution. The reaction mixture was incubated in the dark for 30 min at room temperature; thereafter the absorbance was read at 517 nm with an ultraviolet visible spectrophotometer (Varian-Carey 50). Ascorbic acid and butylated hydroxytolulene (5, 10, 20, 40, and 80 mg/ml), were used as positive controls while the reaction mixture with 50% methanol instead of the extract was used as a negative control. Correction of the colour of the extract was done by taking the absorbance reading of the samples without DPPH and the difference from readings of the samples in the

presence of DPPH. Each sample extract was evaluated in triplicate. Radical scavenging activity (RSA) of plant extracts was determined by the decolouration of the DPPH solution and was calculated according to the following formula:

$$\% \text{ RSA} = (1 - A_E / A_D) \times 100$$

Where  $A_E$  represents the absorbance of the reaction mixture containing the plant extract or positive controls and  $A_D$  represents the absorbance of the DPPH solution only (**KARIOTI et al., 2004**).

#### **6.2.2.2 Ferric-reducing Antioxidant Power (FRAP) Assay**

The ferric reducing power of the plant extracts were evaluated according to **NDHLALA et al. (2013)** and **MOYO et al. (2010)** with slight modifications from **KIM et al. (2009)**. The dried plant extracts together with butylated hydroxytolulene were dissolved in 50% aqueous methanol to a concentration of 10 mg/ml. In a 96-well microplate, 30  $\mu$ l of each extract were serially diluted two-fold with 30  $\mu$ l of distilled water. Forty microliters of potassium phosphate buffer (0.2 M, pH 7.2) and potassium ferricyanide (1% w/v, BDH Biochemicals Ltd. Poole, England) were each then added to the wells. The reaction mixture was incubated at 50 °C for 20 min in the dark after which 40  $\mu$ l of trichloroacetic acid (10% w/v), 150  $\mu$ l of distilled water and 30  $\mu$ l ferric chloride (0.1% w/v, Merck, Darmstadt, Germany) were added to each well. The plate was incubated for a further 30 min at room temperature in the dark and the absorbance measured at 630 nm using an Opsys MR<sup>TM</sup> micro-plate reader (Dynex Technologies Inc., Chantilly VA).

#### **6.2.2.3 $\beta$ -Carotene Linoleic Acid Model System (CLAMS) Assay**

The method used by **MILLER (1971)**, **MILLER et al. (1993)** and modified by **AMAROWICZ et al. (2004)** and **NDHLALA et al. (2014)** with minor changes was used. Sample extracts were dissolved in 50% aqueous methanol to stock solutions of 5 mg/ml concentrations. The process involves autoxidation of  $\beta$ -carotene from the extracts during incubation in a water bath at 50 °C. Ten mg  $\beta$ -carotene, was prepared in 10 ml chloroform in a brown Schott bottle which limits photo-oxidation. The excess chloroform was evaporated under vacuum so that the thin film of  $\beta$ -carotene which remained behind could be turned into a solution by the addition of linoleic acid (200  $\mu$ l) and 2 ml Tween 20. Distilled water (497.8 ml) was aerated and added to a final  $\beta$ -carotene concentration of 20  $\mu$ g/ml. The mixture was oxygen saturated by vigorously shaking it to form an orange coloured emulsion. This emulsion (4.8 ml) was transferred into test tubes with a known concentration (200  $\mu$ l) of the extract at



6.25 mg/ml and standard antioxidant which serves as a positive control. Quercetin, BHT (6.25 mg/ml) or ascorbic acid were selected as positive controls. The reaction mixtures contained a final concentration of 250 µg/ml. The negative control used was 50% aqueous methanol in place of the sample. Tween 20 solution served as a blank for the spectrophotometer. The absorbance for each reaction was measured straight away ( $t = 0$ ) at 470 nm and incubated at 50 °C, and absorbances were recorded for each reaction mixture measured at 470 nm every 30 min for 180 min. The  $\beta$ -carotene bleaching rate was calculated by using the following equation:

$$\text{The rate of } \beta\text{-carotene bleaching} = \ln(A_{t=0}/A_{t=t}) \times 1/t$$

where  $A_{t=0}$  is defined as the absorbance of the emulsion at 0 min and  $A_{t=t}$  is the absorbance at time  $t$ . the average rate of  $\beta$ -carotene bleaching is measured based on the rates at time  $t$ . The calculated average rates used to define the antioxidant activity (ANT) of the extracts and expressed as percentage inhibition of the rate of  $\beta$ -carotene bleaching using:

$$\% \text{ ANT} = (R_{\text{control}} - R_{\text{sample}})/R_{\text{control}} \times 100$$

where  $R_{\text{control}}$  and  $R_{\text{sample}}$  represent corresponding average rates of  $\beta$ -carotene bleaching for the negative control and plant extracts. Antioxidant activity can be further expressed as the oxidation rate ratio (ORR) based on the equation:

$$\text{ORR} = R_{\text{sample}} / R_{\text{control}}$$

Antioxidant activity (AA) was calculated based on the inhibition of coupled oxidation of  $\beta$ -carotene and linoleic acid against the negative control at  $t = 60$  min and  $t = 120$  min using as described by **BRACA et al. (2003)** and **MOYO et al. (2010)**:

$$\% \text{ AA} = [1 - \{A_0 - A_t/A_{00} - A_{0t}\}] \times 100$$

where  $A_0$  is the extract absorbance found at the commencement of incubation,  $A_t$  is the absorbance at time =  $t = 60$  min or 120 min for the extract and  $A_{00}$  and  $A_{0t}$  denotes the absorbance of the negative control (without extract) at the beginning of incubation and at time  $t = 60$  or 120 min, respectively.

The constraint with this assay is the staining of  $\beta$ -carotene at 470 nm which can complicate the interpretation of the results (**PRIOR et al., 2005**). An advantage of this assay is its relevance and application to both hydrophilic and lipophilic environments to identify the pro-antioxidant and antioxidant activity of a compound (**FRANKEL et al., 1993; PRIOR et al., 2005**).

## **6.3 Wound Healing Properties of Plant Extracts**

### **6.3.1 Protein Precipitable Phenolics Capacity Assay as a Wound Healing Model**

The protein-precipitating capacity of the phenolics assay as outlined by **MAKKAR et al. (1999)** was used to determine the in 50% aqueous methanolic plant extracts. Of the 19 plant extracts, 13 were selected to be tested for their wound healing ability from a comparison of all the results of previous assays (Table 6.2). The method is explained below:

### **6.3.2 The Formation of the Phenolic-Protein Complex**

Fifty percent aqueous methanol extracts were added to 2 ml of bovine serum albumin (BSA) solution (with 1 mg BSA/ml acetate buffer), to produce a 3 ml solution (in increasing concentration of 50% aqueous methanol extract vs 50% aqueous methanol as follows: 0.95, 0.90, 0.85, 0.80, 0.75, 0.70 ml of 50% methanol with various concentrations (0.05, 0.10, 0.15, 0.20, 0.25, 0.30 ml) of the plant extracts. This was done in triplicate in centrifuge tubes. A vortex was used to mix the solution and it was then allowed to stand overnight at 4 °C in a centrifuge tube in the refrigerator. The tubes were centrifuged at 1370 x g for 10 min. The supernatant was carefully removed while the precipitate remained at the base of the tube. The precipitate was then constituted with 1.5 ml of 1% sodium dodecyl sulphate (SDS) solution and vortexed until fully dissolved. The dissolved phenolic-protein complex was measured and determined at 510 nm.

### **6.3.3 The Determination of Phenolics in the Phenolic-Protein Complex**

One ml aliquots of soluble phenolic-protein complex dissolved was placed into clean test tubes. A 3 ml solution of SDS-triethanolamine (1% SDS (w/v) and 7% (v/v) triethanolamine in distilled water) was added followed by 1 ml ferric chloride reagent (0.01 M FeCl<sub>3</sub> in 0.1 M HCl).

The measurements were recorded using absorbance readings at 510 nm after 30 min of incubation at room temperature using a UV-Visible spectrophotometer. These readings were taken in triplicate and the average converted into gallic acid equivalents using a standard curve. A linear regression curve was plotted using GraphPad Prism V6 (Graph Pad<sup>R</sup> software Inc. CA). The graph plotted phenolics precipitated as gallic acid equivalents and mg dry plant

extracts. The slope (mg phenolics precipitated/mg plant samples = x) represented the protein-precipitating phenolics in the medicinal plant extracts (MAKKAR, 1999).

#### **6.3.4 The Protein-Precipitating Capacity as a Percentage of Total Phenolics**

Aliquots of 0.05 to 0.30 mg/ml of the 50% aqueous methanol extracts prepared to 1 ml with 1% of SDS and 3 ml of the SDS-triethanolamine solution to which 1 ml ferric chloride reagent was added. Incubation of the solution at room temperature for 30 min was allowed followed by the measurement of absorbance readings at 510 nm. The graph constructed was a linear regression curve with phenolic acid equivalents and mg extract prepared using GraphPad Prism software. The slope of the curve (mg phenolics equivalent /mg extract = y) represented total phenolics. The protein-binding precipitating phenolics were measured as x.

The percentage (%) of total phenolics which can precipitate protein =  $(x/y) \times 100$

### **6.4 Results and Discussion**

The antioxidant activity of the nineteen selected plant extracts are presented below in Tables and Figures after detailed evaluations using the DPPH scavenging assay, the  $\beta$ -carotene linoleic acid (CLAMS), the FRAP models and the protein precipitating/binding assay with reference to the results for each below.

#### **6.4.1 DPPH Assay**

The EC<sub>50</sub> of plant extracts with bold values ( $\mu\text{g/ml}$ ) (Table 6.1) are considered strong DPPH radical scavengers. The effective concentrations at EC<sub>50</sub> were determined and compared to ascorbic acid as a positive control (0.070  $\mu\text{g/ml}$ ). The plant extracts with significantly high DPPH scavenging activity with lower EC<sub>50</sub> than the control were *A. arborescens* leaf (0.043  $\mu\text{g/ml}$ ), *B. frutescens* leaf (0.053  $\mu\text{g/ml}$ ), *B. natalensis* root (0.006  $\mu\text{g/ml}$ ) and *E. autumnalis* root (0.062  $\mu\text{g/ml}$ ). The lower the EC<sub>50</sub> value, the more speedily the DPPH radical was lightened; therefore, the more powerful the antioxidant. The results indicate that at lower concentrations the antioxidant activity for these extracts was higher. All extracts at varying concentrations showed the capacity to release a hydrogen atom or an electron that leads to a reduction in the absorbance of the reaction mixture. The activity varied markedly between the various plant parts with leaf and root extracts generally showing the strongest radical scavenging activity in most plant species tested.

The metal chelation, total antioxidant activity, reducing power and radical scavenging (DPPH) effects as well as those events that are destructive to active oxygen groupings such as the hydroxyl radical, superoxide anion radical and hydrogen peroxide are widely used as antioxidant radicals (SHARMA and BHAT, 2009). DPPH is commonly known as a radical and a trap ("scavenger") for other radicals. The rate of reduction of a chemical reaction when one adds DPPH is a good indication of the sudden changes in nature of the radicals in the reaction (SHARMA and BHAT, 2009). The strong absorption band is around 520 nm. The DPPH radical has a deep violet color in solution, becoming colourless or pale yellow when neutralized. This characteristic feature of DPPH is visually prominent and assists with monitoring any reactions, as well as the number of primary radicals by counting from the initial changes of the optical absorption at 520 nm (ALGER, 1997; COWIE and ARRIGHI, 2008).

#### 6.4.2 CLAMS Assay

In the CLAMS assay, the activity of the antioxidants (ANT - %) was determined using calculations on the basis of the rate of  $\beta$ -carotene bleaching at  $t = 60$  min as each reaction mixture is recorded every 30 min to observe the changes in the reaction mixture. The ORR values (Table 6.1) less than those of butylated hydroxytoluene (BHT) value of 0.232 and transcribed in bold are considered as very potent antioxidants. The percentage activity of antioxidants based on the average proportion of heat-induced  $\beta$ -carotene bleaching was high for several of the tested plant extracts ranging from 29% to 96% relative to 76% BHT standard for the CLAMS assay. In order to reach 50% quenching activity, lower concentrations of plant extracts were seen to be greater than that of the control value of 76%. The lowest activity was 29% recorded from *M. plumbea* root extract. The medicinal plant extracts with strong antioxidant activity included *A. ferox* leaf extract, *B. frutescens* leaf extract, *B. frutescens* root extract, *B. natalensis* leaf extract, *B. natalensis* root extract, *E. autumnalis* leaf extract, *H. limifolia* leaf extract, *H. aethiopicum* leaf extract, *T. riparia* leaf extract, *Z. aethiopica* leaf and *Z. aethiopica* stem extracts.

The extracts from the *A. arborescens* leaf, *B. frutescens* leaf, *B. natalensis* root, *E. autumnalis* root had significant antioxidant activity for DPPH. In addition *A. ferox* leaf extracts, *B. frutescens* leaf and root extracts, *B. natalensis* leaf and root extracts, *E. autumnalis* leaf extracts, *H. limifolia* leaf extracts, *H. aethiopicum* leaf extract, *T. riparia* leaf extract, *Z. aethiopica* leaf and stem extract were significant for the CLAMS model. The extracts from *E. autumnalis* root extracts for DPPH were  $0.062 \pm 0.029$   $\mu\text{g/ml}$  and the the leaf extracts had an ORR of = 0.197. The plant extracts for *H. limifolia* (ORR 0.184), *H. aethiopicum* (ORR 0.175), *T. riparia* (ORR

0.159 ± 0.136) and *Z. aethiopica* (ORR leaf extract 0.138, stem extract 0.075 ) also presented high activity of the antioxidants for the CLAMS assay in relation to the controls (DPPH 0.070 µg/ml, ORR 0.232) respectively. Polyphenol concentrations found in the plant extracts are related to the observed antioxidant activities (Chapter 3). **DALL'AGNOL et al. (2003), AVATO (2005), SUNTAR et al. (2010) and SUNTAR et al. (2011)**, reported on the medicinal use of *Hypericum* species as a balm with unique wound healing properties and also effective against stings and bites. These authors reported that *Hypericum* is a wound herb that healed bruises, opened obstructions, dissolved swellings, and closed up the lips of wounds, sores and for stings and bites. The plant is also known for its antimicrobial activity and that it contains hyperforin and hypericin. According to various researchers, many of the active constituents in *Hypericum* species are polyphenols like flavonoids, phenolics and tannins as well as conjugated phloroglucinol derivatives and benzopyrans (**DALL'AGNOL et al., 2003; MUKHERJEE et al., 2003; AVATO, 2005; SUNTAR et al., 2010; SUNTAR et al., 2011; GUEDES et al., 2012**). *H. aethiopicum* exhibits significant antioxidant activity while *Z. aethiopica* has been screened for its active compounds and found to contain α-linoleic acid, a number of phenylpropanoids and triterpenoids, sterols and lignans (**VAN WYK et al., 2009**).

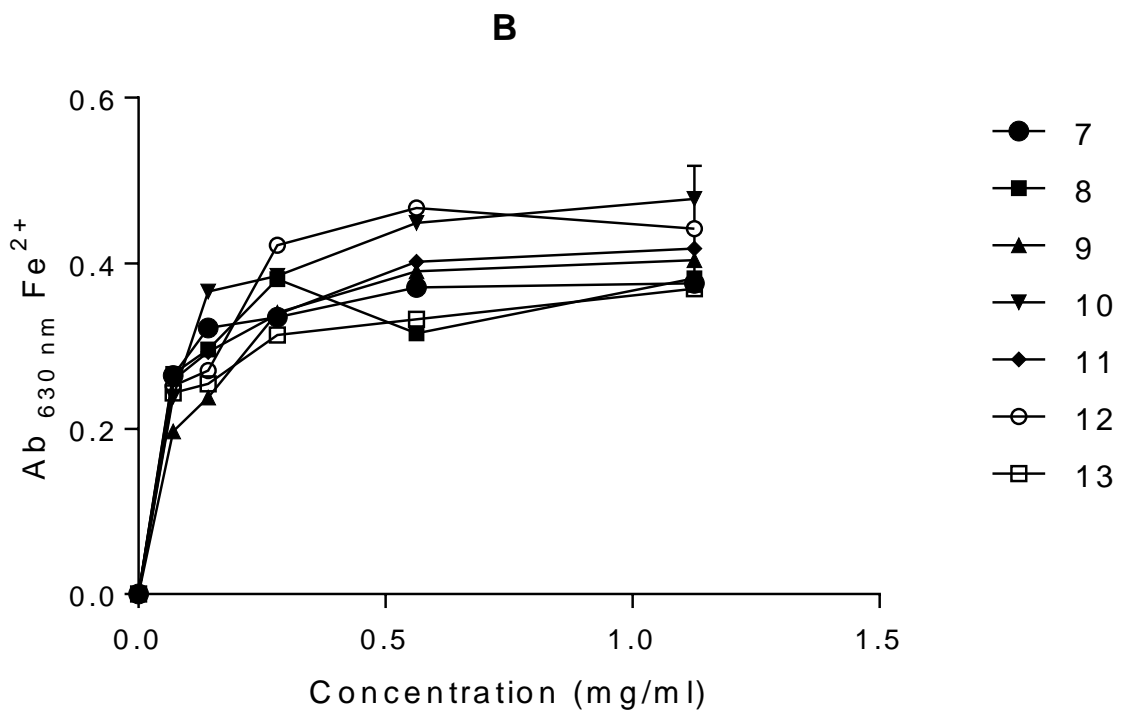
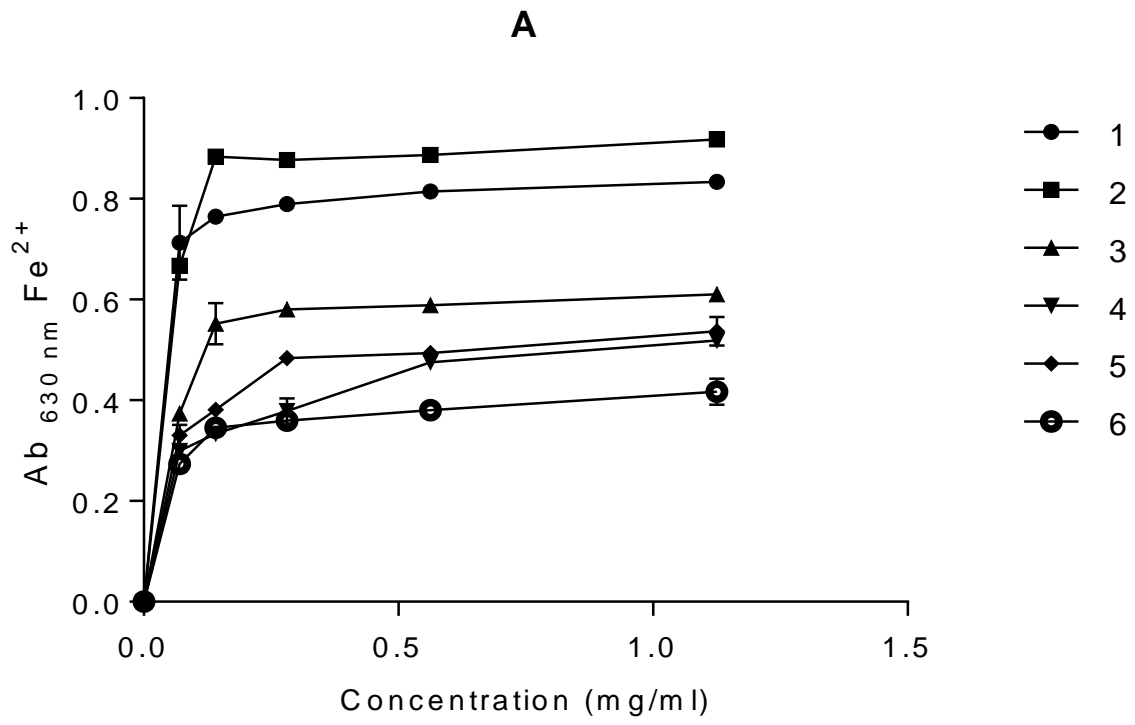
**Table 6.1** Antioxidant activity of nineteen therapeutic plant species used for wound healing and skin disorders in KZN, South Africa as evaluated by the  $\beta$ -carotene linoleic acid models and the DPPH scavenging assay.

Antioxidant Activity			
Plant Species	DPPH EC <sub>50</sub> ( $\mu$ g/ml)	CLAMS	
		ANT (%)	ORR
<i>Aloe arborescens</i> leaves	<b>*0.043 ± 0.009</b>	73.971 ± 7.437	0.260 ± 0.074
<i>Aloe aristata</i> leaves	0.604 ± 0.059	70.299 ± 5.509	0.297 ± 0.055
<i>Aloe ferox</i> leaves	0.273 ± 0.091	87.594 ± 1.778	<b>*0.124 ± 0.018</b>
<i>Bulbine frutescens</i> leaves	<b>*0.053 ± 0.015</b>	91.354 ± 5.191	<b>*0.086 ± 0.052</b>
<i>Bulbine frutescens</i> roots	1.309 ± 0.065	78.372 ± 4.603	<b>*0.216 ± 0.046</b>
<i>Bulbine natalensis</i> leaves	0.601 ± 0.012	96.187 ± 2.751	<b>*0.038 ± 0.028</b>
<i>Bulbine natalensis</i> roots	<b>*0.006 ± 0.000</b>	80.833 ± 1.536	<b>*0.192 ± 0.015</b>
<i>Eucomis autumnalis</i> leaves	2.160 ± 0.139	80.266 ± 0.890	<b>*0.197 ± 0.009</b>
<i>Eucomis autumnalis</i> roots	<b>*0.062 ± 0.029</b>	71.982 ± 1.467	0.280 ± 0.015
<i>Eucomis autumnalis</i> bulbs	0.897 ± 0.112	67.172 ± 0.767	0.328 ± 0.008
<i>Haworthia limifolia</i> leaves	0.300 ± 0.023	81.584 ± 0.325	<b>*0.184 ± 0.003</b>
<i>Hypericum aethiopicum</i> leaves	0.777 ± 0.071	82.468 ± 0.338	<b>*0.175 ± 0.003</b>
<i>Merwillia plumbea</i> leaves	0.258 ± 0.025	58.271 ± 4.922	0.417 ± 0.049
<i>Merwillia plumbea</i> bulbs	0.322 ± 0.031	68.952 ± 2.161	0.310 ± 0.022
<i>Merwillia plumbea</i> roots	1.179 ± 0.020	29.012 ± 0.671	0.709 ± 0.007
<i>Tetradenia riparia</i> leaves	5.531 ± 0.321	84.055 ± 13.620	<b>*0.159 ± 0.136</b>
<i>Tetradenia riparia</i> stems	2.918 ± 0.016	75.573 ± 3.927	0.244 ± 0.039
<i>Zantedeschia aethiopica</i> leaves	1.838 ± 0.189	86.216 ± 3.784	<b>*0.138 ± 0.038</b>
<i>Zantedeschia aethiopica</i> stems	0.652 ± 0.090	92.469 ± 0.655	<b>*0.075 ± 0.007</b>
<b>Ascorbic Acid</b>	0.070 ± 0.010		
<b>Butylated Hydroxytolulene</b>		76.727 ± 6.171	0.232 ± 0.062

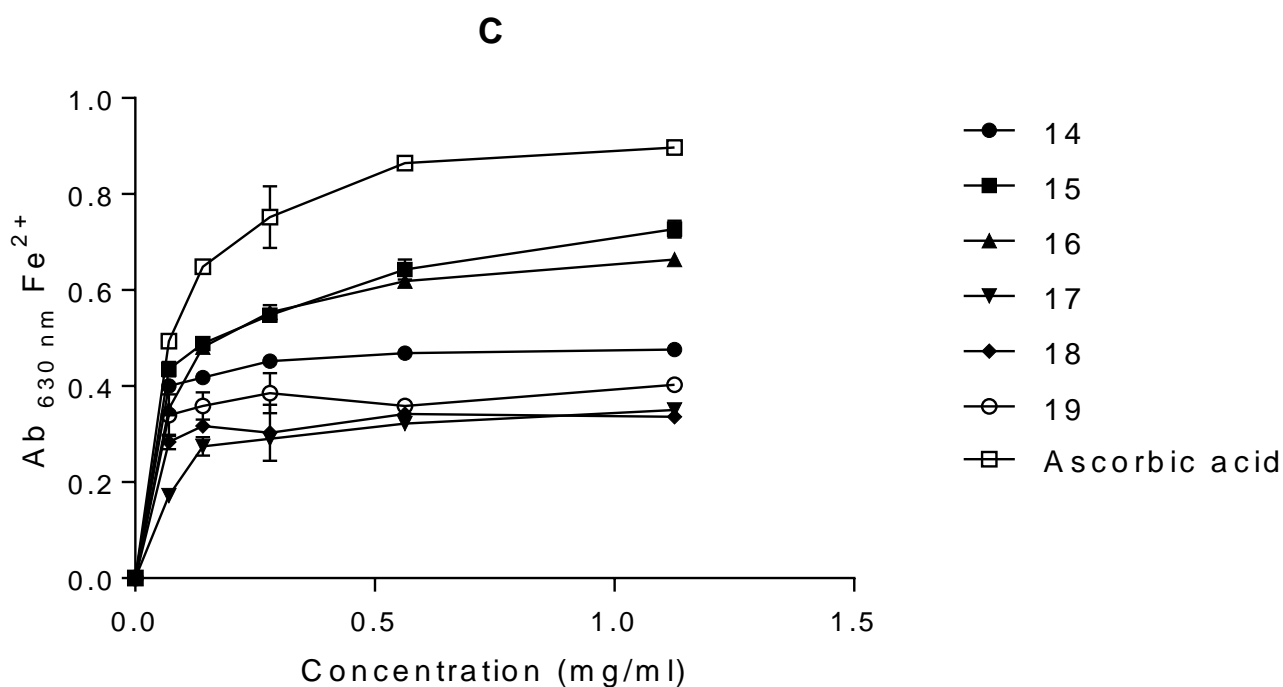
\*Bold indicates the plant extracts with significant antioxidant activity.

### 6.4.3 FRAP Assay

Figure 6.1 represents the capacity of the plant extracts at variable concentration ranges to reduce  $\text{Fe}^{3+}$  solution. Figures 6.1A-C indicate an increase in the absorbance as the concentration levels of the extracts were increased. This depends on the extracts reducing power. The preliminary yellow colour of the reaction mixture changes to various shades of blue and green. The more effective the antioxidant or reductant reducing capacity to increase the ferricyanide complex  $\text{Fe}^{3+}$  to a blue/green ferrous form, the higher the absorbance values at 630 nm recorded. All the plant extracts showed chelating ability at concentrations below 0.1 mg/ml comparable to that of the ascorbic acid control. Phenolic compounds (Chapter 4) can be correlated with the antioxidant and anti-inflammatory activity of the plant extracts. *T. riparia*, popular for its medicinal use in Rwanda has been screened by VAN PUYVELDE et al. (1986) for many active diterpenediols, in this study shows significant antioxidant activity. *M. plumbea* is rich in homoisoflavones, saponins and glycosides (VAN WYK et al., 2009) and the plant extracts are commonly used for wound healing and skin disorders and are also reported for their antimicrobial, anti-inflammatory properties (VAN WYK et al., 2009). Consistent to these findings, the same extracts, showed significant antioxidant activity in this study. The FRAP reducing assay changes the ferric ion to ferric oxide and the activity of the plant extract on the reaction causes a change from a yellow to a blue/green colour. The measuring of this change or the optical density readings for each plant extract provides a good indication of their antioxidant activity. As can be seen in Figures 6.1A-C, All 19 extracts tested in this study showed some degree of antioxidant activity as indicated (Figure 6.1A-C). Of significance, with higher absorbance are *Merwillia plumbea* (0.65), *Tetradenia riparia* (0.6) and the *Aloe* species (0.75-0.84) at 1 mg/ml. The other tested medicinal plant extracts yielded absorbance ranges from 0.24 to 0.57 at the same concentration of 1 mg/ml. For effective wound healing and dermatological conditions, plant extracts with higher antioxidant activity and the capacity to be effective anti-inflammatory agents in the healing process are important attributes.







**Figure 6.1** Ferric-reducing power (FRAP) of nineteen medicinal plant extracts used for skin disorders and wound healing in South Africa. (1) *Aloe arborescens* leaf, (2) *Aloe aristata* leaf, (3) *Aloe ferox* leaf, (4) *Bulbine frutescens* leaf, (5) *Bulbine frutescens* root, (6) *Bulbine natalensis* leaf, (7) *Bulbine natalensis* root, (8) *Eucomis autumnalis* leaf, (9) *Eucomis autumnalis* root, (10) *Eucomis autumnalis* bulb, (11) *Haworthia limifolia* leaf, (12) *Hypericum aethiopicum* leaf, (13) *Merwillia plumbea* leaf, (14) *Merwillia plumbea* bulb, (15) *Merwillia plumbea* root, (16) *Tetradenia riparia* leaf, (17) *Tetradenia riparia* stem, (18) *Zantedeschia aethiopica* leaf, (19) *Zantedeschia aethiopica* stem. Values represent mean  $\pm$  standard error (n = 3).

#### 6.4.4 Protein Precipitation/Binding Assay

The protein binding capacity of the 13 most active plant extracts are presented in Table 6.2. Various levels of protein precipitation capacity were reported and are indicated in the following ranges of scales from 70-100% high, 40-70% moderate, 20-40% low and 0-20% indicating insignificant activity (NDHLALA et al., 2014; NDHLALA et al., 2015). The *H. limifolia* extract exhibited high affinity for protein (83%) while moderate affinity was found in *E. autumnalis* and *B. natalensis* and the activity was either low or insignificant in the rest of the tested extracts. The healing of wounds is a highly intricate physiological development and multiple overlying phases range from formation of granular tissue, inflammation, inhibition of microbial infections and re-epithelialisation, extracellular matrix formation and remodelling of the wounded area or corrective activity at the damaged site (PERINI et al., 2015). Tissue

regeneration due to phenolic compounds is reported in superficial skin, wound and burn healing combined with antimicrobial and antioxidant capacities (**BRUNETON, 1995; LUSEBA et al., 2007**). Phenols have hydroxyl groups that are hydrogen donors and form very strong hydrogen bonds with the proteins particularly the carboxyl groups. High protein affinity or binding capacity occurs when the phenols which are small enough to penetrate inter-fibrillar peptide chains at many positions on the molecules result in the formation of a film that becomes a physical barrier which is very important in the wound healing process. This capacity extends to bonding with microbial cell walls causing inhibition (**MULAUDZI et al., 2012**). There are also adverse effects of protein binding affinity where phenols rich in tannins interact with protein uptake in the mammalian physiological processes and precipitate functional enzymes for biochemical metabolism hence causing a reduction in availability of nutrients and efficacy and value of the medicinal plants. According to **MASONDO et al. (2014)**, *E. autumnalis* has extensive pharmacological potential, exhibits anti-inflammatory, antimicrobial activity and is rich in flavonoids. **VAN WYK et al. (2009)** reported that the presence of triterpenoids may account for the species' wound healing capacity. The plant extracts showed moderate protein binding capacity and good antioxidant activity. *H. limifolia* has a very high protein binding capacity of 83% and good antioxidant ability. A study by **COOPOOSAMY and NAIDOO (2011a)** reported significant antimicrobial activity for *H. limifolia* extracts and the species is known to contain significant amounts of phenolics. However, more research on this plant is necessary. Other than these six significant antioxidant and/or protein-precipitating active plant extracts (*M. plumbea*, *T. riparia*, *E. autumnalis*, *H. limifolia*, *H. aethiopicum* and *Z. aethiopica*), all *Aloe* and *Bulbine* species presented throughout the screening showed moderate protein binding capacity and good antioxidant activity.

Tannins are nature's form of polyphenolic compounds with high molecular weight protein complexes. There are two groups of tannins formed by their structural type variances and include the condensed tannins and the hydrolysable tannins. Tannin quantification method is based on their chemical capacity or their capability to form bonds with protein substrates. Tannin-protein complexes (tannins in the plant extract and the protein, bovine serum albumin) are the basis used to determine protein precipitable phenolics (**NDHLALA et al., 2015**). The ferric chloride assay for total phenolics determines the tannins present in the complex. Measurements done spectrophotometrically will indicate when iron complexes form with phenols to give a pink chromophore. The five medicinal plants mentioned have a consistent pattern of significant activity throughout this study.

**Table 6.2** Protein-precipitating capacity as a wound healing model of phenolic-rich methanolic medicinal plant extracts from KwaZulu-Natal, South Africa.

Protein- precipitating capacity							
Plant species	1	2	3	4	5	6	7
Protein-precipitating phenolics(x)*	11.58 ±0.99	17.69 ± 0.35	10.83 ± 1.87	18.22 ± 0.39	97.82 ± 1.97	3.44 ± 0.12	9.74 ± 0.45
Total phenolics (y)*	76.24 ± 2.72	64.64 ± 1.46	76.11 ± 0.89	66.71 ± 0.52	118.30 ± 3.44	16.68 ± 0.05	20.04 ± 1.21
Protein-precipitating capacity (%)	15.19 ± 0.71	27.39 ± 1.15	14.21 ± 2.29	27.31 ± 0.36	82.71 ± 0.74	20.62 ± 0.79	48.91 ± 5.21
Plant species	8	9	10	11	12	13	
Protein-precipitating phenolics(x)*	2.77 ± 0.04	2.85 ± 0.17	3.24 ± 0.46	4.66 ± 0.25	3.96 ± 0.18	3.91 ± 0.25	
Total phenolics (y)*	5.44 ± 0.08	7.41 ± 0.02	45.15 ± 1.12	30.11 ± 0.79	23.93 ± 0.06	11.87 ± 0.22	
Protein-precipitating capacity (%)	50.87 ± 0.02	38.51 ± 2.43	7.15 ± 0.84	15.46 ± 0.43	16.53 ± 0.73	32.93 ± 1.46	

1 - *Aloe arborescens* leaf, 2 - *Aloe aristata* leaf, 3 - *Aloe ferox* leaf, 4 - *Hypericum aethiopicum* leaf, 5 - *Haworthia limifolia* leaf, 6 - *Merwillia plumbea* leaf, 7 - *Bulbine natalensis* bulb, 8 - *Eucomis autumnalis* bulb/root, 10 - *Tetradenia riparia* leaf, 11- *Eucomis autumnalis* leaf, 12 - *Zantedeschia aethiopica* leaf, 13 - *Bulbine frutescens* leaf. x\* and y\* are the curve slopes (mg phenolics precipitating/mg plant extracts) representative of the protein-precipitating and the phenolic totals in the extracts respectively.

## 6.5 Conclusions

Oxidative damage is key to accelerated pathogenesis in numerous human diseases and natural therapies particularly from phenolic compounds demonstrating good antioxidant and anti-inflammatory activity and many of the plant extract results as discussed are indicative of these medicinal and healing capacities. An extensive evaluation of plant extracts from South African medicinal plants with potent antioxidant activities is required to determine the mechanisms of action that extracts have from various assay models. The antioxidant activities may be enhanced through synergistic effects of the natural products. The study results do present some good anti-inflammatory and antioxidant activities in a number of the selected medicinal plants. The results lend scientific credence in support of the research is particularly on *Aloe* and *Bulbine* species. In addition, the use of *E. autumnalis*, *H. limifolia*, *H. aethiopicum*, *T. riparia* and *Z. aethiopica* as antioxidant medicinal plants for treating skin disorders and for wound healing is observed through the research. The moderate to good protein binding capacity exhibited by some of the tested medicinal plants could be used as a predictive wound healing model for these medicinal plants.

Having considered the outcomes of the results and the relationship of the phytochemicals and antioxidants the following medicinal plants showed a correlation that was significant: *Aloe* species, *B. frutescens*, *B. natalensis* and *H. aethiopicum*, *H. limifolia* and *Z. aethiopica* had high levels of total phenolics, flavonoids and proanthocyanidins correlated to significant antioxidant activity. Further work on the isolation, identification and characterization of the biologically active compounds responsible for the events are necessary. Once isolated the active compounds can further be used to determine and examine the mechanism of activity. The overall results indicate that plant extracts do have the capacity and potential to be used in wound healing and skin care management.

## CHAPTER SEVEN

### GENERAL CONCLUSIONS AND RECOMMENDATIONS

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The study investigated eleven medicinal plant species, used by traditional practitioners, for treating dermatological diseases and wound healing. According to **HOUGHTON et al. (2007)** “it is important in research to note that *in vivo* models tend to give a more accurate reflection of the activity of substances used in traditional medicine, their use in many countries is severely restricted due to economic and ethical concerns and this has led to the widespread use of *in vitro* tests in ethnopharmacological studies”. These tests are extremely useful where the identity of compounds responsible for the biological activity of an extract is being investigated and where limited supplies of material are available, but it is more significant to consider various factors before making over-predictive claims of that activity in one particular system to explain traditional use. The use of only one bioassay gives a very incomplete picture of the effect of the extract on the whole system involved. A symptom may be due to a number of disease states and, consequently, a variety of mechanisms may serve as targets for bioassays. Similarly, it is very rare for there to be only one target for a particular disease so a variety of test systems must be employed. Numerous test systems are used to assess plants and other plant materials having the capacity of being useful in wound-healing, diabetes, cancer and in the treatment of cognitive decline associated with old age. In addition, consideration must be given to factors such as the absorption into the body and the metabolism of any plant substances present, either to decrease or increase the effect of the activity on health care.

Despite the extensive health care practices using medicinal plants in KwaZulu-Natal and South Africa, a very limited number of science-based studies, nor clinical trials have been published to date recognizing the practical use of indigenous flora for skin care and wound healing. The science fraternity needs to increase the number of studies and investigations which could lead to clinical trials being conducted with preparations from the diverse species of plant taxa that have been used traditionally over many centuries. The mechanisms of action by which plant extracts and their active compounds exert their bioactivity need to be further investigated. In addition, the undiscovered uses of plants will open up potential areas for research. Further exploration of currently used preparations may well result in the discovery of dermatological health care products and effective wound care and healing creams. Ethnobotanical studies show

that the use of traditional plants for dermatological use and wound healing is still prevalent in many communities across the globe. The preservation of local culture, heritage and the practice of traditional health care represents important strategies for the sustainability of popular knowledge of plants and must be maintained. There is growing interest in South Africa in the health benefits of indigenous plants.

The antimicrobial screening of the selected plants was extensive and the outcomes of the basic disc diffusion assay served as a basis for the selection of the plants for the minimum inhibitory concentration (MIC) assays. Those plant extracts that were tested against the seven Gram-negative bacteria and seven Gram-positive bacteria as well as the four fungal strains were based on known dermatological effects. Many of the plant extracts expressed good inhibitory potential against a widespread range of the bacteria. The outcomes from disc diffusion screening for the extracts whose inhibition activity zones were 10 mm and more were further investigated using the MIC assay. Interestingly, the same plant parts screened for both bacteria and fungi were similar. The plant part extracts showing these outcomes were *Z. aethiopica* leaf, *B. frutescens* leaf, *E. autumnalis* bulb and root, *T. riparia* leaf, *H. limifolia* leaf, *B. natalensis* leaf and root, *M. plumbea* root and leaf extracts of *A. aristata*, *A. ferox*, *A. arborescens* and *H. aethiopicum*. The MIC values were important in highlighting minimum concentrations at which medicinal plant extracts were most effective. The following extracts yielded effective inhibitory concentrations of between 0.31 to 0.63 mg/ml for antibacterial activity; *Aloe* species, *Bulbine* species, *T. riparia*, *H. aethiopicum*, *E. autumnalis*, *Z. aethiopica* and *M. plumbea*. The antifungal minimum concentrations were also significant for the above-mentioned plant extracts.

In the phytochemical assessment of total phenolics, flavonoids and proanthocyanidins of the screened medicinal plants; seven of the abovementioned medicinal plants are significant and the results were high for the concentration of phenols for all three groups of tested compounds. These are the *Aloe* species across all three assays (4.53 to 4.69 mg GAE/g DW for total phenolics; 3.41 to 5.28 mg CTE/g DW for flavonoid content; 3.51 to 5.28 µg LCE/g DW for proanthocyanidin content), *H. limifolia* (4.46 mg GAE/g DW for total phenolics, 3.11 mg CTE/g DW for flavonoid content, 3.04 µg LCE/g DW for proanthocyanidin content) and *H. aethiopicum* (3.35 mg GAE/g DW for total phenolics, 4.56 mg CTE/g DW for flavonoid content, 9.85 µg LCE/g DW for proanthocyanidin content), *M. plumbea* 2.04 µg LCE/g DW for proanthocyanidin content and *Z. aethiopica* 2.13 mg CTE/g DW for flavonoid content.

Cytotoxicity screening on all plant extracts for both Brine shrimp lethality assay and the MTT assay indicated no cytotoxic effects with LC<sub>50</sub> values more than 2 mg/ml and larger than 1 mg/ml respectively. Further investigations are necessary as many of the selected medicinal plants are toxic and misuse may result in mortality. Although the results detect no cytotoxicity in the selected plants, some authors have found some to be poisonous; further intensive toxicity investigations would be recommended using water and other solvents in order to determine minimum toxicity levels. Traditional medicine has been used for treating diseases over centuries, scientific studies have revealed that many of them are potentially toxic. The use of herbs may mimic, magnify or oppose the effect of drugs and should therefore be used with care and proper consultation and information should be provided by health care practitioners when mixing herbs and pharmaceutical drugs as interaction with most drugs is not known. Other examples of identified herb-drug interactions include bleeding when warfarin is combined with ginkgo (*Ginkgo biloba*), mild serotonin syndrome in patients who mix St John's wort (*Hypericum perforatum*) with serotonin-reuptake inhibitors and potentiation of oral and topical corticosteroids by liquorice (*Glycyrrhiza glabra*) and soluble bres (including guar gum and psyllium) which can decrease the absorption of drugs. An example of South African herb-drug interactions is the use of devil's claw with the use of warfarin resulting in purpura. In addition, *H. hemerocallidea* and *S. frutescens* showed a negative interaction with antiretroviral medication, thus patients may be at risk from treatment failure, viral resistance, or drug toxicity.

The anti-inflammatory IC<sub>50</sub> concentrations for the nitric oxide assay was less active and the results were greater than 100 µg/ml for four of the medicinal plant extracts, namely the three *Aloe* species and *H. limifolia* but significant for all other extracts with values ranging from 13.99 to 29.35 µg/ml. In the LOX assay, in addition to the above four plants, the findings for *M. plumbea* had less activity whilst five of the medicinal plant extracts, *Bulbine natalensis*, *E. autumnalis*, *H. aethiopicum*, *T. riparia* and *Z. aethiopica* were effective agents with IC<sub>50</sub> values below those of the quercetin control and ranged from 3.55 to 9.52 µg/ml.

Antioxidant screening for all medicinal plants revealed significant activity with the FRAP assay. In the DPPH assay model, EC<sub>50</sub> values showed significant activity for the three *Aloe* species, two *Bulbine* species, *E. autumnalis*, *H. limifolia*, *H. aethiopicum*, *T. riparia* and *Z. aethiopica* while the CLAMS results were similar to those of DPPH for the same plant species. The protein-precipitating capacity as a wound healing model was high for *H. limifolia* leaf at 82.7% while the other ten medicinal plants (*A. arborescens* - 15.2%, *A. aristata* - 27.4%, *A. ferox* - 14.2%, *H. aethiopicum* - 27.3%, *M. plumbea* - 20.6%, *B. natalensis* - 32.9%, *T. riparia*

- 7.15%, *E. autumnalis* - 15.5%, *Z. aethiopica* - 16.5% and *B. frutescens* - 32.9%) had moderate to low values. Overall, the results from this multi-dimensional medicinal plant research study provide extensive information on the potential of 11 plant species on wound healing and skin diseases.

Plant extracts are associated with several antimicrobial properties including anti-inflammatory and antioxidant. The use of bioactive extracts or phytochemicals from a variety of plants contributes to public health care. The interaction of the antioxidants and the free radicals is an important process for maintaining good health, and reducing age-related conditions and diseases. Those free radicals generated in excess of the normal may accumulate and outweigh the protective effects of antioxidants. Science needs to strengthen the multidisciplinary research mechanisms to support the evaluation of medicinal plants and effectively facilitate collaboration between traditional and conventional health practitioners. National regulatory frameworks on indigenous medicinal practices and various strategic plans for policy implementation on traditional health care and medicines are integral to enhancing traditional medicine research and the continual outcomes of science based evidence on the efficacy, quality and safety of traditional medicines. The use of National, WHO and International research protocols and guidelines are essential. Intensifying the aspects of traditional medicine into higher education curricula, syllabi and training programmes to bring traditional medicines into Primary Health Care systems could be effective strategies that can provide breakthroughs in understanding traditional health care management systems. The defence of indigenous knowledge and intellectual property rights and the protection and conservation of plant species that are at risk of extinction in South Africa is crucial. Actively promoting collaboration across research centres and amongst partners who are strong in the field of ongoing research on traditional medicinal plants could accelerate development of traditional medicine into the mainstream health system.



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