

**AN INVESTIGATION INTO THE ANTIBACTERIAL ACTIVITIES
OF
MEDICINAL PLANTS TRADITIONALLY USED IN THE EASTERN CAPE
TO TREAT SECONDARY SKIN INFECTIONS
ASSOCIATED WITH BURN WOUNDS.**

By

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CONTENTS

	Page
ABSTRACT	i
ACKNOWLEDGEMENTS	iii
LIST OF ABBREVIATIONS	iv
LIST OF TABLES	vii
LIST OF FIGURES	ix
CHAPTER 1 INTRODUCTION	
1.1 Overview	1
1.2 Statement of the problem	4
1.3 Aim	4
1.4 Objectives	4
CHAPTER 2 LITERATURE REVIEW	
2.1 TRADITIONAL MEDICINE	
2.1.1 Traditional African herbal medicine	5
2.1.2 Herbalism and primary health care	7
2.1.3 Traditional herbalism and westernised biomedicine	8
2.2 MEDICINAL PLANTS	
2.2.1 Importance of traditional medicinal plants	10
2.2.1.1 Ethnobotanical surveys	12
2.2.1.2 Phytomedicine and phytochemistry	13
2.2.1.3 Conservation of medicinal plants	15
2.2.2 Drug-herb interactions	16
2.2.3 Medicinal plants under investigation	18

2.2.3.1	<i>Bulbine frutescens</i>	21
2.2.3.2	<i>Leonotis leonurus</i>	21
2.2.3.3	<i>Melianthus major</i>	22
2.2.3.4	<i>Zantedeschia aethiopica</i>	23
2.3	TOPICAL BURN WOUND INFECTIONS	
2.3.1	Skin: Overview	25
2.3.2	Skin related infections	27
2.3.2.1	Wound infection and inflammation	27
2.3.2.2	Burn wound infection	29
2.3.2.3	Pathogens associated with burn wound infections	31
2.3.3	Bacteria selected for investigation	33
2.3.4	Antibiotic resistance	36

CHAPTER 3 METHODOLOGICAL JUSTIFICATION

3.1	Medicinal plants	38
3.2	Extraction of medicinal plants	39
3.3	Assays for antibacterial analysis	39

CHAPTER 4 METHODS AND MATERIALS

4.1	Plant selection and preparation	42
4.2	Plant extraction and traditional preparations	42
4.2.1	Plant extraction	42
4.2.2	Traditional preparations	45
4.3	Bacteria and growth conditions	45
4.4	Antibacterial assays	
4.4.1	Microtitre plate assay	46
4.4.2	Agar dilution assay	49
4.4.2.1	Agar plate preparation	50
4.4.2.2	Agar plate inoculation	51
4.4.3	Standard agar plate count technique	52

CHAPTER 5	RESULTS	
5.1	Microtitre plate assay	
5.1.1	Undiluted plant extract concentrations	53
5.1.2	Visible representation of MIC's in the microtitre plates	55
5.1.3	Antibacterial activity screening and MIC determination	56
5.1.4	Results of antibacterial activity screening of plant extracts	60
5.1.5	Standard antimicrobial sensitivity patterns of Gram-positive and Gram-negative bacteria	65
5.1.6	Plant extract dilutions that displayed antibacterial activity	69
5.1.7	Minimal inhibitory concentrations of plant extracts	70
5.1.8	The number of bacterial strains inhibited by the plant extracts	77
5.1.9	Antibacterial activity of traditional medicinal plant preparations	78
5.2	Agar dilution assay	
5.2.1	Antibacterial activity screening and MIC determination of plant extracts	80
5.2.2	Antibacterial activity of traditional plant preparations	83
5.3	Standard agar plate count technique	84
CHAPTER 6	DISCUSSION AND CONCLUSION	86
	REFERENCES	96

ABSTRACT

Traditional medicine has a long history of being used for treating various ailments ranging in severity. Although traditional medicine has typically been the health care for the poorest levels of society, there is a worldwide growth in popularity. The growing popularity of traditional medicine, termed the green boom, may be ascribed to people taking a more holistic approach to maintain their health.

Traditional medicine is widely used on a regular basis by 70% of South Africans. Various indigenous medicinal plants are used for the preparation of traditional herbal medicine. These plants are mostly indigenous to the regions where it is used.

In this study four medicinal plants (*Bulbine frutescens*, *Leonotis leonurus*, *Melanthus major* & *Zantedeschia aethiopica*) that are traditionally used in the Eastern Cape region for treating burn wound infections, were collected for investigation. The *in vitro* antibacterial activity of these plants was tested against different bacterial strains of eight different bacteria. The bacteria used in this investigation included bacterial strains of four Gram-positive bacteria, *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *E. faecalis*, *S. pyogenes* and four Gram-negative bacteria, *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* and *P. mirabilis*.

Traditional preparations as well as three different extracts (methanol, aqueous & acetone) of the plants were used for *in vitro* antibacterial activity testing. The microtitre plate assay and agar dilution assay were used for determining the antibacterial activity of the traditional preparations and plant extracts against the different bacterial strains. In the microtitre plate assay the antibacterial activity was tested using the bacterial growth indicator, INT and a microtitre plate spectrophotometer to determine the minimal inhibitory concentrations of the plant extracts and traditional preparations.

The microtitre plate assay was used for testing the antibacterial activity of the plants against the bacterial strains of five bacteria, *S. aureus*, MRSA, *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*. The bacterial strains of the three bacteria, *S.*

pyogenes, *E. feacalis* and *P. mirabilis* were not compatible with the microtitre plate assay using INT and spectrophotometric readings to determine bacterial inhibition. Therefore the agar dilution assay were used as an alternative method for determining the MIC's of the plant extracts against the bacterial strains of these bacteria.

The initial plant extract concentration in the microtitre plate assay differed with the different plant extracts in the microtitre plate assay. Acetone followed by methanol extracted the highest plant extract concentrations with the different medicinal plants. *M. major* followed by *L. leonurus* produced the highest plant extract concentrations following extraction with the different extraction solvents. Consequently the acetone extract of *M. major* had the highest plant extract concentration before serial dilution in the microtitre plate assay. Uniform plant extract concentrations were tested in the agar dilution assay.

The methanol extract followed by the acetone extract of the plants gave the highest antibacterial activity against the different bacterial strains. The extracts of *M. major* followed by *L. leonurus* inhibited the highest number of bacterial strains in the microtitre plate assay and the extracts of *B. frutescens* inhibited the lowest number of bacterial strains.

The acetone and methanol extracts of *M. major* were the only extracts that displayed antibacterial activity in the agar dilution assay. The bacterial strains of *P. mirabilis* were the only bacteria that were inhibited using this method. The bacterial strains of *S. pyogenes* and *E. feacalis* were not inhibited at any of the plant extract concentrations in the agar dilution assay.

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LIST OF ABBREVIATIONS

A

Ave average

B

BA blood agar

BSA Body Surface Area

C

°C degree Celsius

cfu/ml colony forming units per millilitre

Conc Concentration

D

DMSO dimethylsulphoxide

DST Diagnostic Sensitivity Test

F

Fig Figure

G

g gram

H

hr hour

I

INT *p*-iodonitrotetrazolium

M

Max maximum

mg milligrams

MH Mueller-Hinton

MIC minimal inhibitory concentration

Min minimum

ml millilitre

MRSA methicillin-resistant *Staphylococcus aureus*

N

NHLS National Health Laboratory Services

nm nanometre

No. number

P

PBS Phosphate Buffered Saline

PNPG *p*-nitrophenyl glycerol

R

rpm revolutions per minute

U

UPE University of Port Elizabeth

UV Ultra Violet

V

VRE Vancomycin Resistant Enterococcus

W

WHO World Health Organisation

LIST OF TABLES

TABLES	Page
TABLE 1 Summary of the selected medicinal plants used for traditional treatment of skin diseases	24
TABLE 2 Summary of the selected test bacteria and their microbial characteristics	35
TABLE 3 Schematic representation of the extraction procedure	44
TABLE 4 The number of bacterial strains tested for antibacterial activity	45
TABLE 5 Average concentration of the undiluted plant extracts in the microtitre plate wells	54
TABLE 6 Antibacterial activity screening of the medicinal plants with the different bacterial strains	58
TABLE 7 Screening results of medicinal plants for antibacterial activity	63
TABLE 8a Standard antimicrobial sensitivity patterns of Gram-positive bacterial strains selected for MIC determination	67
TABLE 8b Standard antimicrobial sensitivity patterns of Gram-negative bacterial strains selected for MIC determination	68
TABLE 9a Representation of the extract dilutions where the MIC's of medicinal plants was obtained for the bacterial strains	73
TABLE 9b Minimal inhibitory concentrations of medicinal plant extracts for antibacterial testing	74

TABLE 10	The lowest and highest MIC's for each medicinal plant extract in the microtitre plate assay	75
TABLE 11	Dilutions that displayed antibacterial activity for traditional plant preparations	79
TABLE 12	Antibacterial activity of plant extracts in the agar dilution assay	81
TABLE 13	Higher concentrations of the acetone and methanol extracts of <i>M. major</i> tested for antibacterial activity	82
TABLE 14	Antibacterial activity of traditional plant preparations on agar plates	83
TABLE 15	Colony forming units (cfu/ml) of microtitre plate bacterial suspensions	85

LIST OF FIGURES

FIGURES	Page
FIGURE 1 Display the appearance of the medicinal plants selected for antibacterial investigation	20
FIGURE 2 Representation of the main protective skin layers and the depth of burn injury	26
FIGURE 3 Schematic representation of the different steps performed for assessing the antibacterial activity of the medicinal plants	41
FIGURE 4 Layout of the 96-well microtitre plate: dimensions and test suspensions	47
FIGURE 5 Representation of the calculation performed to obtain the bacterial inhibition	48
FIGURE 6 Lack of INT reduction by the three bacteria	49
FIGURE 7 Reduced swarming of <i>P. mirabilis</i> on DST agar plates supplemented with PNPG	51
FIGURE 8 Plant extract concentrations in the undiluted microtitre plate wells	55
FIGURE 9 Representations of MIC's for acetone extracts of <i>M. major</i> in the microtitre plates	56
FIGURE 10 The lowest MIC values of the different medicinal plants in the microtitre plate assay	76
FIGURE 11 The highest MIC values of the different medicinal plants in the microtitre plate assay	76

FIGURE 12	Number of bacterial strains inhibited by the plant extracts in the microtitre plate assay	77
FIGURE 13	Antibacterial activity of fresh and stored extracts of <i>M. major</i>	80
FIGURE 14	Antibacterial activity of acetone extracts of <i>M. major</i> against <i>P. mirabilis</i> at higher concentrations	82
FIGURE 15	Representations of bacterial colony forming units/ml on agar plates	85

CHAPTER 1

INTRODUCTION

1.1 Overview

Medicinal plants are still being used today with increasing popularity for treating or preventing various ailments. These plants form an integral part in the healing processes of traditional medicine used by traditional healers.

Traditional healers commonly referred to as inyanga's (herbalists) have a broad knowledge of medicinal plants and concentrate on the healing properties of these plants. Medicinal plant usage forms the backbone in many rural communities for treating ailments with varying severity (Van Wyk *et al.*, 1997).

Although medicinal plants form a central component in traditional medicine and serve as a basis for primary health care in various communities, many medicinal plants have not been scientifically researched and investigated. Reports on the scientific investigation of medicinal plants in the various types of infection may enhance utilization of efficacious medicinal plant remedies (Rakungira, 2002).

Medicinal plants play a vital role in the treatment of skin ailments both, traditionally and in the cosmetic industry as many skincare products are supplemented with plant extracts. Commercially, the cosmetic skincare industries are taking advantage of various scientifically researched medicinal plant extracts that are beneficial to the skin for inclusion into skincare products. In South Africa as well as in other countries the *Aloe* species, *Centella* plant species and various other medicinal plant products have become synonymous with the maintenance of a healthy and beautiful skin (George *et al.*, 2001, Van Wyk & Gericke, 2000).

Many people in this day and age are aware and try to avoid the harmful effects of some synthetic ingredients in skincare products by choosing a more holistic

approach to maintaining the well being of their skin. Some individuals with a damaged skin may be affected psychologically influencing their self-confidence if their skin is profusely damaged for prolonged periods. The emotional trauma of a profusely damaged skin caused by infective agents may encourage the use of various skincare products. The simultaneous use of different skincare products may be viewed as a plight of desperation to improve the skin condition.

There are skincare products containing extracts from medicinal plants that claim to reduce scarring, eczema and wound infections based on their unique composition of traditional medicinal plant extracts. Commercial formulations include one of the most studied wound-healing plants, *Centella asiatica*, that promote the formation of structural tissue components and increased keratinisation of the epidermis (Van Wyk & Gericke, 2000). The *Aloe* species that are mostly native to East and South Africa are widely known and used as a topical agent for its wound healing properties in treating burns and traumatic wounds. *Calendula* plant extracts with its reported anti-inflammatory properties have been used for treating wounds to promote repair and healing (<http://content.nhiondemand.com/dse/consumer/>).

According to ethnobotanical surveys, skin infections are one of the most common ailments treated traditionally in the Eastern Cape region using medicinal plants (Matsiliza & Barker, 2001; Grierson & Afolayan, 1999b). The topical nature of infected wounds allow for observation in progression and regression when using medicinal plants therapeutically. Economic factors such as long travelling distances to reach medical facilities contribute to the use of medicinal plants for treating common ailments, thereby eliminating long waiting periods (Pillay *et al.*, 2000). Regression of minor wound infections may be prevented and slowed if long periods before treatment are avoided and immediate therapeutic action is taken.

Conventional medicine, although helpful, are beyond the means of many people in rural communities where there is increasing rates of unemployment and rising medical costs. The inappropriate use of conventional medicine especially antibiotics should be discouraged as it may encourage antibiotic resistance.

In the rural communities where there is limited access to medical facilities, people should be empowered through education on the proper use of medicinal plants as first-aid remedies (Bodeker, 2001; Rabe & van Staden, 1997).

Burn injuries cause mechanical disruption of the skin, which allows environmental microbes to invade the deeper tissues. Infections remain the leading cause of death among patients hospitalised for burns (Schwarz, 2001).

Research showed that the most common infections occurring in burnt children are burn wound infections and catheter-associated septicaemia (Rodgers *et al.*, 2000). Internationally, the incidence of wound infections is higher with overcrowded burn units and when patients have less access to immediate wound debridement or antimicrobial therapies (Schwarz, 2001).

There are various organisms involved in causing infection of burn wounds such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* (Schwarz, 2001) and beta haemolytic *Streptococcus* group A (Macfarlane *et al.*, 2001, p.162; Rodgers *et al.*, 2000). Certain strains of staphylococci and streptococci have been documented to defy multiple antibiotics and are therefore extremely difficult to control (Levy, 1998).

Medicinal plants in this study were selected based on the traditional ethnobotanical uses in the Eastern Cape region for treating wound and burn wound infections. The medicinal plants may be used fresh or as a traditional herbal preparation for topical application on the affected wound area. Different extracts of the four selected medicinal plants, *Bulbine frutescens*, *Leonotis leonurus*, *Melianthus major* and *Zantedeschia aethiopica* were used for assessing the antibacterial activity against different strains of bacterial isolates from burn patients. Besides the plant extracts traditional herbal preparations were also prepared for detecting the *in vitro* antibacterial activity of the four plants against clinical isolates of burn wound pathogens.

1.2 Statement of the problem

Infections are the major life-threatening complication of burn injuries. Secondary bacterial infections in burn wounds, impede healing of the wounds (Damjanov, 1996) and have many complications such as immune suppression (Schwarz, 2001). Colonization of resistant species such as *P. aeruginosa* (Pruitt *et al.*, 1998), development of methicillin-resistant *S. aureus* infection and death due to secondary sepsis (Haruff, 1994, p.45) may also complicate burn wound infections. Countries need expensive drugs when resistance becomes a clinical problem (Levy, 1998).

1.3 Aim

- Establish scientific validity of the selected medicinal plants, traditionally used for treating skin conditions in the Eastern Cape region, by conducting research into the antibacterial activity of these plants.

1.4 Objectives

- Collecting indigenous medicinal plants occurring in the Eastern Cape Province for *in vitro* antibacterial testing against different clinical strains of bacteria commonly causing secondary infection in burn wounds.
- Screen the different plant extracts and the traditional preparations of medicinal plants for *in vitro* antibacterial activity.
- Report the minimal inhibitory concentration (MIC) of the screened medicinal plant extracts using the selected antibacterial assays.
- Add information and knowledge to the scientific research done on indigenous medicinal plants used traditionally in South Africa especially in the Eastern Cape region.

CHAPTER 2

LITERATURE REVIEW

2.1 TRADITIONAL MEDICINE

2.1.1 Traditional African herbal medicine

The utilization of medicinal plant remedies in preventing or curing various ailments were the sole source of ensuring human welfare until the development of chemistry and organic compound synthesis in the 19th century (Kong *et al.*, 2003, <http://www.world.std.com/~krake/>). Traditional African medicine is a holistic discipline involving an extensive use of indigenous herbalism in combination with aspects of African spirituality (George & van Staden, 2000).

A third of the global population lacks access to essential medicine and up to 80% of the population in Africa uses traditional medicine for primary health care (http://www.who.int/medicines/library/trm_polpers_eng.pdf). Traditional herbal healing is widely practiced throughout South Africa and an estimated 70% of South Africans regularly use medicinal plant based medicine. Therefore quick evaluation of its safety, efficacy, quality and standardization, is of prime importance in preserving our heritage and the ongoing utilization of medicinal plants (WHO, 2002; Eloff, 1998b).

Traditional medicine is defined by the World Health Organization (WHO) as “including diverse health practices, approaches, knowledge and beliefs incorporating plant, animal and/or mineral based medicines, spiritual therapies and exercises, applied singularly or in combination to maintain well being, as well as to treat, diagnose or prevent illness” (http://www.who.int/medicines/library/trm_polpers_eng.pdf). There have been many definitions and interpretations for defining the practice of herbalism with its extensive therapeutic usage of traditional medicinal plants.

South Africa has an estimated 200 000 indigenous traditional healers who are commonly known as inyanga's (herbalists) and sangomas (diviners) which forms the backbone of primary health care in Africa (Van Wyk *et al.* 1997). Most tribes in South Africa use medicinal plants and other material that is mostly indigenous to the region where they practice for preparation of traditional remedies (Matsiliza & Barker, 2001).

Some people especially in the rural communities rely completely on the healing properties of traditional medicinal plants provided by herbalism. Other people refer to herbalism only on certain occasions as alternatives to allopathic medicine or in combination with other therapeutic regimes. The use, dosage and preparation methods of medicinal plants are usually provided by traditional herbalists (inyanga's) in rural communities (Matsiliza & Barker, 2001).

For many Africans living in rural communities where there is irregular income and rising medical costs, therapeutic herbalism has become a dependant way of health care (Stanley, 2004). The popularity of herbalism is not restricted to rural communities, but there has been a worldwide upsurge in the interest and preference to traditional herbalism. The worldwide revival of herbalism may be attributed to people taking a more holistic view in maintaining human well being (Kong *et al.*, 2003).

In the Eastern Cape people still depend to a large extent on traditional herbal medicine for the treatment of various diseases and ailments. A study revealed 38 plant species, which are commonly used for the treatment of wounds by traditional healers and the indigenous people of the Eastern Cape province (Grierson & Afolayan, 1999b).

Indigenous health care traditions are centred on the particular skills of an individual practitioner. Training of an individual practitioner may occur as a result of apprenticeship to an experienced herbalist, spiritual ancestral calling or through informal learning from a close family member (Bodeker, 2001, http://www.conserveafrica.org/medicinal_plants.rtf). Prominent features of traditional healers include a deep personal involvement in the healing process, the protection of therapeutic knowledge by keeping it secret and a deeply rooted knowledge of the

uses of medicinal plants (Matsiliza & Barker, 2001; http://www.conserveafrica.org/medicinal_plants.rtf).

Some studies in the Eastern Cape region have documented the different ways in which medicinal plants are utilized and prepared in order to prevent the loss of invaluable age old knowledge (Matsiliza & Barker, 2001; Grierson & Afolayan, 1999a). Traditional indigenous herbal knowledge serves as a source of many known and untapped contributions to an improved maintenance of health care (Rabe & van Staden, 1997).

2.1.2 Herbalism and primary health care

Traditional medicine systems have typically been the primary health care of the poorest levels of society. A large portion of the population still relies on traditional practitioners and local medicinal plants for satisfying their primary health care needs (Bodeker, 2001; <http://www.who.int/inf-fs/en/fact134.html>; Grierson & Afolayan, 1999b). According to George & van Staden (2000) despite the increasing popularity of medicinal plants in South Africa the importance of traditional medicine for primary health care is not always recognized.

Herbalism is not only practiced as an alternative health option but also to meet the growing urban demand for traditional medicine (Matsiliza & Barker, 2001).

Traditional herbal medicine is the major and in some cases the only source of health care available in many rural communities. Local people treat themselves with various medicinal plants at an early stage of disease, at a low cost and conveniently replacing the indiscriminate use of unprescribed drugs (Rabe & van Staden, 1997; http://www.conserveafrica.org/medicinal_plants.rtf).

Although herbs appear to be harmless and beneficial to everybody, numerous toxic compounds were found in remedies such as deadly nightshade and jimson weed (Dautra). Some side effects associated with medicinal plants include the reduced availability of oral contraceptives and theophyllin with the simultaneous intake of St. John's Wort extracts (Dharmananda, 2000). In China, incorrect dosage of a traditional remedy containing a herb used for short term treatment led to a dozen

deaths, heart attacks, and strokes as a result of improper long term dietary aid administration (<http://www.who.int/mediacentre/factsheet/fs134/en/print.html>).

The prices of pure isolated substances from plants are beyond the financial resources of most people in developing countries (George & van Staden, 2000). Isolated compounds from medicinal plants might have the potential to be promoted to such an extent that it may result in a similar outcome as some synthetic miracle compounds. The inappropriate use of some synthetically produced drugs have facilitated the development of antimicrobial resistance, with its life threatening consequences (Lewis, 1995). Therefore it is essential that the correct dosage, safety, stability, efficacy and other important factors pertaining to plants used indigenously are thoroughly researched and documented (Eloff, 1998b).

It was recommended by the WHO in 1996 to test and document the different standards defining the identity, purity, and potency of plants used medicinally in the form of a plant monograph. Thereby the monograph serves as a revised carbon copy of traditional herbal knowledge for future generations (<http://www.sahealthinfo.org/traditionalmeds/aboutuwc.htm>).

2.1.3 Traditional herbalism and westernised biomedicine

The WHO defines health as “a complete state of physical and mental well being, and not merely as an absence of disease or infirmity” (<http://www.thelancet.com/search/search.isa>). In contrast to westernised biomedicine where the patient is viewed as a sum of organ systems, neurophysiological hydraulics and where only the affected parts of the body are treated, traditional healers take a more holistic approach, looking at the whole body (physical, mental and spiritual) (<http://www.thelancet.com/search/search.isa>; http://www.conserveafrica.org/medicinal_plants.rtf).

The quality of herbal medicine is influenced by factors such as the location of growth and correct identification of collected medicinal plants (www.who.int/medicines/library/trm_polpers_eng.pdf). Besides the variability of some medicinal plant therapies, it has been used for millennia standing the test of time as useful therapeutic remedies. Although conventional medicine is regulated

under strict quality control measures, the high cost of these medications make them inaccessible to people in poor communities. The widely available, inexpensive herbal remedies is a favourable choice of medication and treatment in impoverished communities with economic and transport burdens.

The number of traditional health practitioners per capita is substantially higher than that of trained medical personal per capita in countries such as Ghana, Swaziland and South Africa (http://www.conserveafrica.com/medicinal_plants.rtf; Bodeker, 2001). The traditional healer-to-patient ratio is estimated between 1:200 and 1:400 compared to 1:20 000 for conventional general practitioners in Africa (Stanley, 2004; <http://www.i-sis.org/GSFTM.php>). The WHO estimated that herbalism is 3-4 times more commonly practiced than conventional medicine worldwide (<http://www.thelancet.com/search/search.isa>).

The use of traditional medicine as primary health care is encouraged as a result of the following:

- healers knowing the sociocultural background of people,
- healers are highly respected and experienced in their work,
- economic considerations,
- variable effects of many modern drugs,
- long waiting times at hospitals,
- unmotivated poorly trained hospital staff and
- shortage of health professionals, particularly in rural areas (Bodeker, 2001; http://www.conserveafrica.com/medicinal_plants.rtf; <http://www.who.int/inf-fs/fact134.html>; Rakungira, 2002).

In many societies both traditional and modern health systems exist and normally people consult both systems for different reasons and during different stages of their disease (Bodeker, 2001; http://www.conserveafrica.com/medicinal_plants.rtf). A study of medicinal plants used in Lesotho indicated the one-way referral on certain occasions from traditional healers to modern doctors (Shale *et al.*, 1999).

In countries such as Vietnam and China the modern and traditional health systems are integrated at the level of medical education and practice (Bodeker, 2001). In

South Africa there has been interest of integrating the different health systems, such as the 48-bed hospital in Kwa-Mhlanga founded by a traditional African healer that combines the different aspects of traditional African, Western and other complementary healing practices (Helwig, 2000).

The formation of an integrated traditional and modern health care system as well as fostering collaboration and teamwork among all categories of health care workers may lead to an improved system of maintaining human welfare (Bodeker, 2001; <http://www.who.int/mediacentre/factsheet/fs134/en/print.html>).

2.2 MEDICINAL PLANTS

2.2.1 Importance of traditional medicinal plants

South Africa is the third most biologically diverse country on the earth, with 80% of more than 18 500 vascular plant species found nowhere else (Siegfried, 1989; World Conservation Monitoring Centre, 1992). Medicinal plants also called “muthi plants” forms the basis of traditional herbal medicine (Fyhrquist *et al.*, 2002; <http://www.world.std.com/~krahe/>). South Africa has a vast array of medicinal plants (<http://www.thelancet.com/search/search.isa>) used in the treatment of various diseases on a regular basis (Lin *et al.*, 1999). There are believed to be about 27 million consumers of medicinal plants in South Africa (Mander, 1998).

Although South Africa contains about 10% of the worlds plant diversity, relatively little work has been done on the medicinal plants from this region (George *et al.*, 2001). There is limited research and investigation concerning the therapeutic potential of medicinal plants (Lin *et al.*, 1999).

Scientific investigation has led to the development of various plant-based medicine, with a worldwide contribution to health care. Information concerning the identification, uses and preparatory methods of medicinal plants has a long history of being documented mostly in the form of ethnobotanical surveys.

The utilization and identification of medicinal plants as therapeutic agents have various facets, aimed at improved health care. Medicinal and poisonous plants have always played an important role in the African society, for containing substances with healing properties (Fyhrquist *et al.*, 2002; <http://www.world.std.com/~krahe/>). Active antimicrobial agents such as polygodal, anethole, safrole and cryptoline form part of the secondary metabolites that serve as sources of defence agents against microorganisms (Fabry *et al.*, 1998).

Many modern medicines have their origins in plants, which have been used for millennia in treating various ailments and standing the test of time as sources of potent and powerful drugs (Bodeker, 2001; Ahmad *et al.*, 1998).

These medicinal plants form an important foundation in various ethnobotanical studies and phytochemical analysis in the different traditions worldwide. Medicinal plants and their derivatives contribute to more than fifty percent of all drugs used worldwide (Van Wyk *et al.*, 1997; Kong *et al.*, 2003).

The therapeutic use of medicinal plants has gained recognition in many rural communities as effective remedies for treating various ailments ranging from acute skin conditions to chronic bronchitis (Ahmad *et al.*, 1998). Therapeutic utilization of medicinal plants is not only limited to the rural population but also to meet the growing demand in urban communities (Matsiliza & Barker, 2001). The easy availability of low cost medicinal plants may attribute to the therapeutic acceptance of medicinal plants in rural communities (http://www.indmedplants-kr.org.THE_NEED_FOR_THE.HTM).

The popularity of medicinal plants may be seen in the estimated 20 000 tonnes of over 700 medicinal plants traded annually in South Africa (George *et al.*, 2001). The increased medicinal plant utilization has led to growing concerns in the sustainability of medicinal plants and the possibility of herb-drug interactions due to indiscriminate use.

2.2.1.1 Ethnobotanical surveys

Ethnobotanical surveys are of paramount importance in the correct identification, utilization and application of the therapeutic potential of medicinal plants from the various traditions. The surveys have an early history for recording and documenting the folklore remedies handed down from generation to generation. Some countries like Africa, India and China are treasure houses of traditional and local knowledge that is the fruit of centuries sometimes millennia of experience in plant use (http://www.indmedplants-kr.org/THE_NEED_FOR_THE.HTM). Ethnobotanical surveys may be viewed as a form of preserving the invaluable age-old knowledge of the curative medicinal plant properties.

The use of ethnobotanical surveys assisting the selection of plants for investigation has led to the discovery of an estimated three quarters of pharmaceutically active plant derived components. Surveys of indigenous traditional knowledge is cost effective in terms of time and capital when it is used as the basis for drug exploration (George *et al.*, 2001; Cox & Balick, 1994).

Medicinal plants may have more than one common name because of the different preparatory methods in different communities for treating different ailments (Grierson & Afolayan, 1999b). Ethnobotanical surveys may aid in the identification of the different applications, uses and common names of frequently used medicinal plants in the various communities and traditions. The correct identification of medicinal plants with its common name and various preparatory methods in surveys may assist in preventing improper utilization of medicinal plants.

The incorrect identification of medicinal plants may result in serious side effects even death (Helwig, 2000), therefore ethnobotanical surveys is useful not only as a means of knowledge preservation but also as a reference guide. An ethnobotanical survey done by Grierson & Afolayan (1999) identified leaves followed by stem bark as the most frequently used plant parts for treating wounds in the Eastern Cape province.

2.2.1.2 Phytomedicine and phytochemistry

Approximately 350 plant species of an estimated 3000 higher plant species commonly used in South Africa for medicinal purposes has undergone chemical investigation (George *et al.* 2001). The popularity of medicinal plants accompanied with the limited phytochemical analysis, has led to increased research in the safety and efficacy of these medicinal plants.

Medicinal plants have a wide reputation for containing active components that serve as phytomedicinals and valuable therapeutic agents for treating disease.

Modern medicine rely heavily on the active compounds of plants, be they natural or synthetic such as secondary metabolites in higher plants which serve as defence agents against microorganisms (<http://www.world.std.com/~krahe/>, Fabry *et al.*, 1997). At least 130 plant drugs used worldwide are either isolated compounds extracted from higher plants or were modified synthetically (George & van Staden, 2000).

According to Kong *et al.* (2003) among the recent 25 best-selling drugs in the world, 30% of the drugs originated from natural products. Some South African phytomedicine that have gained worldwide popularity include devil's claw, African potato, buchu, aloe products and rooibos tea (George *et al.*, 2001).

Recent developments in the traditional medicine sector include anti-diabetic and anti-cancer formulations from *Sutherlandia* under the brand name Healer's Choice jointly developed by Rand Afrikaans University and Pharmacare (George & van Staden, 2000). Up to 90% of phytomedicinals available in South Africa are exotic European formulations (George *et al.*, 2001). According to George & van Staden (2000) there is a growing need to promote the development and marketing of indigenous medicines, some of which are more efficacious than imported formulations.

Research and development of plant based drugs is time consuming, resource intensive and full of uncertainties (George & van Staden, 2000). The ethnobotanical approach to sampling plants for research are based on traditional knowledge of

medicinal plants and offer strong clues to the biological activities of those plants (Cox & Balick, 1994).

Research organizations and universities need to work in collaboration with mutual objectives on the research of medicinal plants for widespread screening of these plants (Spjut, 1985). At the University of Cape Town, research groups like South African Traditional Medicine Group, have been directed towards the development of a database containing information to avoid repetitive research (George & van Staden, 2000).

Pharmacognosy that includes the identification, extraction and application of specific plant constituents responsible for specific therapeutic actions may assist the efficacious use of some medicinal plants. Morphine, codeine, quinine, aspirin and Taxol are examples of some well-known plant derived, standardized drugs (Van Wyk *et al.*, 1997). The discovery of the novel antimalarial agent, artemisin from the plant species of *Artemisia* is useful for the treatment of chloroquine-resistant malaria cases without causing side effects (Kong *et al.*, 2003). The active constituents in Digitalis plant leaves were concentrated to deliver standardized, set doses of the active compound, digoxin, used for treating heart failure (Hanrahan, 2001).

Not all synthesized compounds are equal to the active compounds naturally occurring and derived from plants. Synthetic vincristine has an inferior efficacy as appose to the same active compound occurring naturally in the plant, *Cathranthus roseus* (http://www.indmedplants-kr.org/THE_NEED_FOR_THE.HTM).

The future of phytochemical research in South Africa has a promising outlook in phytomedicinal discovery. One recent finding is that the root bark extract of *Bobgmonia madagascarensis* was shown to be more potent than established anti-fungals against *Candida albicans* (George *et al.*, 2001). Martini and Eloff (1998) isolated at least fourteen antibacterial compounds from the South African species, *Combretum erythrophyllum*, with some isolated compounds illustrating higher activity than chloramphenicol and ampicillin.

2.2.1.3 Conservation of medicinal plants

The revival of medicinal plant usage as easily accessible therapeutic agents, has led to an increase demand for medicinal plant resources. The medicinal plant trade has become highly commercial with an estimated plant trade of R62 million per annum in KwaZulu-Natal (http://www.kznwildlife.com/muthi_trade.htm). An estimated 20 000 tones of over 700 medicinal plant species are traded annually in the herb market industry of South Africa (George *et al.*, 2001).

The high demand of medicinal plants in South Africa has led to over 10% of more than 20 000 plant species being threatened with a decreased availability and listed in the South African Red Data books (Goldring, 1999; George & van Staden, 2000). These plants may harbour active components that are needed to overcome or reduce the problems experienced with some modern drugs. Despite the potential medicinal plants have as sources for new antimicrobial agents, some plants without proper scientific investigation and documentation are faced with extinction.

Some early conservation measures used by traditional herbalists include the prevention of ring barking a tree, avoiding the complete removal of plant roots as well as preventing repetitive plant harvesting from the same site. These early conservatory methods were believed to maintain plant potency and the balance between plant removal and plant re-growth in the wild (Matsiliza & Barker, 2001).

Despite some of the informal conservation measures employed by traditional herbalists, destructive harvesting of some plants is still visible. Plant collectors that are unappreciative and unfamiliar with the correct harvesting techniques carry out destructive harvesting techniques such as ring barking and the complete removal of roots that prevent plant re-growth for future use (Matsiliza & Barker, 2001). As a result many plants are protected under law governing the harvesting of the medicinal plants in KwaZulu-Natal (Diederichs *et al.*, 2002).

The increasing popularity and high demand of some medicinal plants have made them objects of extensive overexploitation. *Ocotea bullata* (black stinkwood) and *Warburgia salutaris* are some of the many medicinal plant species that are under

threat of decreased availability and in need of immediate conservation (George *et al.*, 2001). The Durban municipality in KwaZulu-Natal has a medicinal plant nursery, Silverglen, which cultivates about 120 at risk plant species (<http://www.botany.uwc.ac.za/envfacts/facts/traditional.htm>).

Industrialization and overpopulation are contributing factors to the decreased availability of medicinal plants that harbour antimicrobial agents (Fabry *et al.*, 1998). Increasing modernization that is accompanied by knowledge erosion and acculturation by the current generation poses a more serious threat than resource erosion in conserving medicinal plants (Matsiliza & Barker, 2001; http://www.conserveafrica.org/medicinal_plants.rtf).

Although ethnobotanical information of medicinal plants is not always passed on by word of mouth, this knowledge can be preserved in the form of ethnobotanical surveys and records. Educating people about the importance of medicinal plants and encouraging them to appreciate our valuable plant resources may prevent destructive harvesting.

2.2.2 Drug-herb interactions

In this section the terms 'herbal remedies or herbs' will refer to remedies with a medicinal plant basis or composition and 'prescription medicine or drugs' will refer to synthetically manufactured conventional drugs with set limits on dosages.

People often perceive herbal remedies as natural products or food and assume these remedies to be free from side effects (Ko, 1999). Herbal remedies are complex mixtures of more than one active ingredient (Ernst, 2000). According to Fugh-Berman (2000), interactions between drugs and herbs may increase or decrease the pharmacological or toxicological effects of components.

The revival of medicinal plants as therapeutic remedies encouraged the movement of herbs and plants from the garden to the medicine cabinet. Not only does herbal remedies, like conventional medicine have the power to harm as well as help, but taken simultaneously with prescription medicine, can result in potentially harmful

interactions (Peoples Medical Society, 2000). With pharmaceutical drugs, the therapeutic and toxic drug dose limits are well established whereas herbal remedies are largely unregulated forming part of dietary supplements.

There are many facets contributing to the occurrence of drug-herb interactions and its undesired outcomes. It may be in the event of trying to speed up the healing process by mixing prescription medicine and herbal remedies that an unintentional adverse drug-herb interaction occurs.

Some herbal remedies may decrease the plasma concentration (bio-availability) of certain drugs by inducing the enzyme responsible for metabolising the drug. A documented example of such an herbal remedy is St. John Wort extracts that decrease the bio-availability of certain drugs taken concurrently, below effective levels (Williamson, 2003; Ruschitzka *et al.*, 2000; Ernst, 1999).

In this study it should be noted that although the medicinal plant remedies are used externally, it should not be excluded from the possibility of drug-herb interactions. Adverse skin reactions may occur due to the concomitant use of antibiotic creams and medicinal plant remedies. Plants are of relevance to dermatology for both their adverse and beneficial effects on skin.

Approximately a third of all traditional medicines are used for treating wounds and skin disorders, compared to 1-3% of modern drugs. Adverse effects of plants on skin include irritant contact dermatitis, caused by irritant chemicals in plant sap and phytophotodermatitis due to skin contamination with furocoumarin-containing plants and subsequent ultra-violet (UV) light exposure (Mantle *et al.*, 2001).

Although the term 'herbal' are mostly perceived to be something that is safe and only of plant origin, there has been reports of heavy metal contamination, misidentification and addition of pharmaceutical substances to herbal remedies (Fugh-Berman, 2000). Therefore it is important to correctly identify and investigate the herb suspected of causing an adverse effect. Inherently toxic herbs, improper preparation techniques and quality control are some of the factors contributing to adverse effects associated with herbs (Ko, 1999).

The issues relating to drug-herb interactions may affect those that consider using herbs and prescription medicine simultaneously and are unaware of the possibility of interactions. Although some of the drug-herb interactions are overestimated, it may be safer to consider its possibility and respect the medicinal properties of both. According to Dharmananda (2000), there is a possibility that drugs and herbs may interact, if the herb has a therapeutic effect similar to that of a drug.

The lack of knowledge of the possibility of drug-herb interactions should be addressed through education that the simultaneous use of prescription medicine and herbal remedies may have undesirable outcomes. The potential for herbs and conventional medicine to interact unfavourably or favourably may depend on the mixture and concentrations. Those using herbal remedies or supplements should be encouraged to record and report their herbal usage to physicians, before additional medication is administered (Fugh-Berman, 2000).

2.2.3 Medicinal plants under investigation

In this study four medicinal plants (*Bulbine frutescens*, *Leonotis leonurus*, *Melianthus major* & *Zantedeschia aethiopica*) were selected on the basis of their ethnobotanical importance in literature and to people, especially in the Eastern Cape region. Although the selected medicinal plants may be used for treating various ailments (Table 1), this study focuses on justifying the traditional use in treating topical wound infections.

Matsiliza & Barker (2001) indicated with a survey done in the Eastern Cape region that the most commonly used traditional remedies was for pain relief and treatment of skin diseases. The survey also indicated the ease of local people in identifying medicinal plants for personal therapeutic uses. Medicinal plants play an important role in the belief system and well being of many people especially those that rely mainly on traditional plant remedies for primary health care. It is important and necessary that the strong believe and use of medicinal plants be accompanied by scientific validation (Matsiliza & Barker, 2001).

Some of the medicinal plants investigated in this study are known to be poisonous and may have harmful or fatal outcomes if its ingested or taken internally (Figure 1.). *Zantedeschia aethiopica* and *Melianthus spp.* form part of the poisonous plant list in Southern Africa (Van Wyk *et al.*, 2002). *Zantedeschia aethiopica* causes mechanical irritation of the mouth when ingested and *M. major* is poisonous for internal consumption and may have fatal outcomes. The reported traditional uses (Table 1) of these plants for treating skin infections employ the external application of the plant remedies (Grierson & Afolayan, 1999b; Van Wyk *et al.*, 1997).



Bulbine frutescens
(balsemkopiva)

(Not poisonous with ingestion)

Leonotis leonurus
(wilde dagga)

Medicinal plants

Melianthus major
(kruidjie-roer-my-nie)

(Poisonous with ingestion)

Zantedeschia aethiopica
(vark lelie)



FIGURE 1 Display the appearance of the medicinal plants selected for antibacterial investigation (Adapted from Van Wyk *et al.*, 1997; Van Wyk & Gericke, 2000)

2.2.3.1 *Bulbine frutescens*

Bulbine frutescens is an aloe-like plant with succulent green leaves containing a clear gel inside the leaves that is released upon breaking or cutting the leaves (Figure 1). *Bulbine frutescens* belongs to the family Asodelaceae and is distributed in the eastern and northern parts of South Africa. The flowering *B. frutescens* has clusters of flowers on thin stalks, which may be yellow or orange in colour.

In this study the *B. frutescens* samples collected for investigation had clusters of orange-coloured flowers (Figure 1). Traditionally *B. frutescens* are known as 'balsemkopiva' (Afrikaans) of which the fresh leave sap or gel are mainly used for treating a variety of skin conditions including burns, wounds, rashes, cuts, abrasions and boils (Table1) (Van Wyk & Gericke, 2000; Watt & Breyer-Brandwijk, 1962).

The anthraquinone knipholone isolated and identified from the fresh bulbs of *B. frutescens* displayed no antibacterial activity against *P. aeruginosa* and *S. aureus* in a disc-diffusion assay (Van Staden & Drewes, 1994). The glycoproteins, aloctin A and aloctin B, found in the leave gel of *B. frutescens* are likely to be responsible for its healing effect. These glycoproteins have also been isolated from *Aloe* species that is widely used in treating skin irritations, bruises and burns (Van Wyk *et al.*, 1997).

2.2.3.2 *Leonotis leonurus*

Leonotis leonurus belongs to the family Lamiaceae with a wide natural distribution over large parts of South Africa especially along the coast. The flowering *Leonotis leonurus* has characteristic clusters of bright orange, hairy flowers on the branch ends (Figure 1). *Leonotis leonurus* has long, narrow leaves with an aromatic odour, especially upon crushing of the leaves.

Leonotis leonurus is commonly known as 'wilde dagga' and 'wild dagga' in Afrikaans and English respectively. Traditional preparations (decoctions & infusions) of *Leonotis leonurus* have a wide variety of medicinal uses externally and internally. Traditionally fresh or dried leave decoctions are used externally to treat a variety of

skin conditions and internally for coughs, colds, influenza, bronchitis, high blood pressure and headaches (Table 1) (Van Wyk *et al.*, 1997; Hutchings, 1996).

Leonotis leonurus contains a volatile oil and an unusual diterpenoid namely marubiin. The actual pharmacological effect of marubiin in *Leonotis* species is not known (Van Wyk *et al.*, 1997).

2.2.3.3 *Melianthus major*

The genus *Melianthus* is restricted to Southern Africa with *Melianthus major* and *Melianthus comosus* mainly used in a similar manner for medicinal purposes. *Melianthus major* belongs to the family Melianthaceae with a wide distribution in South Africa, mainly in the dry interior. The multi-branched *M. major* has greyish-green leaves with ruffled edges and characteristic large clusters of dark purplish-red flowers at the branch end (Figure 1).

Decoctions and poultices of *M. major* leaves are widely used in the treatment of skin conditions (Table 1) and other ailments. The Afrikaans and English common names of *M. major* are 'kruidjie-roer-my-nie' and 'giant honey flower', respectively. Traditionally fresh leaf and root preparations are mainly used externally for medicinal purposes due to its toxicity for internal consumption (Van Wyk *et al.*, 1997; <http://www.plantzafrica.com/plantklm/melianthusmajor.htm>).

Leaf poultices and decoctions of *M. major* are directly applied to impetigo, septic wounds, sores, ringworm, bruises, backache and rheumatic joints. Traditionally dried and powdered leaves are applied directly to sores and open burn wounds. The powdered, dried leaves are reported to relieve pain, retract the wound and facilitate healing (Van Wyk & Gericke, 2000).

The toxicity of *Melianthus* species for internal consumption is due to the presence of heart glycosides, such as melianthusigenin in *Melianthus* species. Although nothing appears to be known about the wound healing properties, the medicinal value of *Melianthus* may be due to triterpenoids in the leaves and roots (Van Wyk *et al.*, 1997).




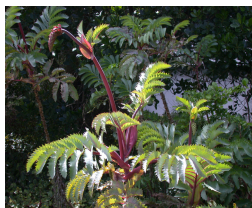
2.2.3.4 *Zantedeschia aethiopica*

This evergreen plant with its large fleshy leaves forms part of the family, Araceae. *Zantedeschia aethiopica* has characteristic large, glossy, dark green leaves and white lily-like ‘flowers’ during the flowering season (Figure 1). The genus *Zantedeschia* mainly found in South Africa is restricted to the African continent. *Zantedeschia aethiopica* is distributed over a large part of South Africa and its coastal regions.

Traditionally *Zantedeschia aethiopica* are also known as the ‘varklelie’ (Afrikaans) and ‘arum lily’ (English). Although *Z. aethiopica* are not truly toxic, the needle shaped calcium oxalate crystals may cause mechanical irritation, if taken internally (Van Wyk *et al.*, 2002). Traditionally the fresh leaves are warmed and used as plasters for treating skin conditions (Table 1) (Grierson & Afolayan, 1999b).

No pharmacologically interesting compounds are known from *Zantedeschia* species. The wound healing effect of *Z. eathiopica* may be due to the protective and moisturising effect of the naturally glossy leaves (Van Wyk & Gericke, 2000).

TABLE 1 Summary of the selected medicinal plants used for traditional treatment of skin diseases

FAMILY: Medicinal plants	Local names: Afrikaans (A), English (E)	Part used and traditional application:	Traditional uses:	References:
ARACEAE: <i>Zantedeschia aethiopica</i> 	Arum lily (E), Vark lelie (A)	Heated fresh leaves as plasters.	Warmed leave plasters for dressing sores, boils, wounds and minor burns.	Grierson & Afolayan, 1999b; Van Wyk & Gericke, 2000.
ASPODELACEA: <i>Bulbine frutescens</i> 	Balsemkopiva (A)	Fresh leave juice/gel externally.	Wounds, burns, rashes, itches, ringworm, herpes and cracked lips	Van Wyk <i>et al.</i> , 1997; Watt & Breyer- Brandwijk, 1962.
LAMIACEAE: <i>Leonotis leonurus</i> 	Wild dagga (E) Wilde dagga (A)	Leave decoctions externally.	Boils, eczema, skin diseases, itching & muscular cramps.	Van Wyk <i>et al.</i> , 1997; Watt & Breyer- Brandwijk, 1962; Hutchings, 1996
MELIANTHACEAE: <i>Melianthus major</i> 	Giant honey flower (E), Kruidjie-roer-my-nie (A)	Leave decoctions externally.	Septic wounds, sores, bruises, backache, rheumatic joints.	Van Wyk <i>et al.</i> , 1997; Watt & Breyer- Brandwijk, 1962

(Pictures taken from <http://gardening.worldonline.co.za/1144.htm>;
http://ag.arizona.edu/pima/gardening/aridplants/Bulbine_frutescens.html)

2.3 TOPICAL BURN WOUND INFECTIONS

2.3.1 Skin: overview

Skin covers the external surface of the body and is one of the largest organs in surface area and weight (http://www.skinhealing.com/2_2_skinburnscars.shtml; Totoro & Grabowsky, 1996, p124). Skin forms part of the non-specific host defences and functions as a mechanical barrier to the surrounding environment and against microbial invasion (Nester *et al.*, 2004, p. 374). Injuries to the skin (cuts, punctures, burns, bites etc.) provide an entry route for pathogens to infect the skin and underlying tissues. The skin serves many functions and externally it often defines our emotional well being through appearance.

The skin contains its own population of beneficial commensal bacteria that resides on the skin surface. *Diphtheroids*, *Staphylococcus* spp. (coagulase negative), *Micrococcus* spp., *Bacillus* spp. & fungi form part of the normal microbial population found on the skin surface (Nester *et al.*, 2004, p. 535; Packham, 1998, p. 321). Normal skin flora has a protective function by competitively excluding harmful microorganisms. Many microorganisms (normal flora) in the correct place are beneficial to humans, however the same microorganism may be harmful, even deadly in the wrong place (Packham, 1998, p. 309). *Staphylococcus epidermidis*, a normal commensal of the skin, may have fatal consequences if it breaches the skin integrity and enters the blood circulation.

Normal skin flora in combination with regular maintenance of skin hygiene may limit the colonization of harmful pathogens on the skin surface. According to Packham, (1998, p. 322) maintenance of the skin in a good condition should employ the removal of pathogenic microorganisms without affecting the normal flora. Excessive or misuse of some germicidal cleansers and soaps may deplete the commensal microorganisms and select bacterial resistance (Levy, 1998). The depletion of normal skin flora and loss of natural skin moisture may facilitate the colonization of harmful microorganisms on the skin surface (Aleksun & Levy, 2001; Levy, 1998; Packham, 1998, p.316).

The skin is composed of two main protective portions overlying the subcutaneous layer mainly:

- **epidermis** – superficial, thinner portion composed of layers epithelial cells and embedded keratin (Figure 2). Contains no blood vessels and regenerates without scar tissue if its damaged. The body reproduces the protective epidermis every 30 days.
- **dermis** – deeper thicker portion composed of tightly woven connective tissue and are responsible for skin elasticity and strength (Figure 2). Contain blood vessels and heal with scar formation (Totora & Grabowski, 1996, p 126; http://www.skinhealing.com/2_2_skinburnscars.shtml).

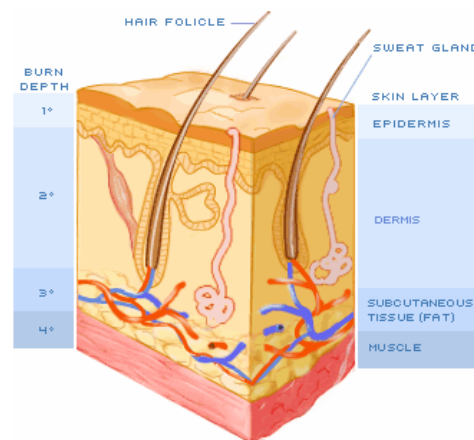


FIGURE 2 Representation of the main protective skin layers and the depth of burn injury

(Taken from http://www.skinhealing.com/2_2_skinburnscars.shtml).

The severity of skin damage is related to the degree of invasion into the epidermis and dermis layers (Ward & Saffle, 1995) (Figure 2). Skin damage may originate from physical injury such as burns, microbial agents (bacterial, viral, fungal, parasitic) or a combination of the two such as burn wound infections.

The skin is a complex organ that is able to resist infection based on its properties and regenerate by healing if tissue damage occurs. Tissue damage resulting from physical agents, microbial agents or a combination of the two may initiate an inflammatory response that is aimed at restoring tissue function (Nester *et al.*, 2004,

p. 386). The process of wound healing is complex and dynamic involving a series of physiological phases. Healing is the final stage of response to tissue injury (Macfarlane *et al.*, 2001).

2.3.2 Skin related infections

A disruption of the skin integrity forms a favourable site for colonization and infection with microorganisms. Invading microorganisms may originate endogenously from the individual and/or exogenously from the environment and fomites. A failure of wound healing may lead to infection, resulting in experiences of increased trauma and treatment costs (Bowler *et al.*, 2001; O'Dell, 1998).

Skin infections are among the most popular conditions still treated traditionally by using medicinal plants. Especially in the Eastern Cape people to a large extent rely on medicinal plants to cure various ailments, including skin diseases (Matsiliza & Barker, 2001; Grierson & Afolayan, 1999a).

Intact skin acts as a protective barrier to invading microorganisms and ensures a healthy balance of skin flora. Accidental cuts, abrasions and burns in combination with contaminants may predispose infection if left untreated (O'Dell, 1998). Diagnosis of infection is primarily based on the presence of clinical signs and symptoms. The topical nature of skin infections allows visible observation and monitoring for progression or regression especially when self-medicating (Kingsley, 2001).

The difficulty associated with reaching modern medical facilities for common health issues enhances the reliance on traditional herbal remedies. The reliance on traditional herbal healing methods for treating skin conditions has a deep-rooted history in many cultures (Harsha *et al.*, 2003).

2.3.2.1 Wound infection and inflammation

The development of wound infection is not a new phenomenon and is an ongoing problem for many people. Open wounds serve as an entry route for contamination

with invading microorganisms. Besides delayed wound healing of infected wounds, it is associated with increased discomfort, hospital stay and health care costs (Kingsley, 2001). Infected wounds scar more severely and are associated with prolonged restoration. The risk of systemic infection and even death is also associated with infected wounds (Ward & Saffle, 1995).

Infection denotes an inflammatory process caused by disease producing organisms. An inflammatory response is initiated by large quantities of different cell types entering the wound, which is ultimately aimed at restoring homeostasis (Collier, 2004). Any agent (microbiologic, physical or chemical in origin) causing cell injury, induces the release of inflammatory mediators (Crowley, 1997, p.82). The classic signs characterizing inflammation, namely redness, pain, swelling, heat and tenderness are mostly self-limiting and subsides with elimination of the harmful agent (Crowley, 1997, p.81).

Virulence and dosage of an invading organism as well as the defence mechanisms of the body has an influence on the outcome of infection. Infection is promoted when the body's defence mechanisms are compromised in combination with highly virulent microorganisms (Crowley, 1997, p.83). The outcome of infection provokes a series of local and systemic host responses (Bowler *et al.*, 2001). Infection is characterised by an enhanced severity of the signs of inflammation such as increased exudates, pus and odour (Kingsley, 2001).

A multitude of microbial and host factors are involved in the development of a wound infection. According to Bowler *et al.* (2001) the type, size, site, and depth of a wound, the host immune status and the virulence of invading microorganisms are some of the factors influencing the progression to an infected wound state. Prolonged or contaminated operations, smoking, malnutrition and inadequate antibiotic prophylaxis are factors elevating the risk of surgical wound infection (Kingsley, 2001).

Infection not only delays healing but may also leads to tissue necrosis in wounds. The presence of necrotic tissue and large amounts of exudates in wounds encourage microbial proliferation and infection (Kingsley, 2001; Collier, 2004). Unhygienic

techniques especially when handling wounds play an important role in predisposing the development of infection (Collier, 2004). According to Collier (2004) microorganisms gain access to a wound in a number of ways:

- self-contamination: patients' skin or gastrointestinal tract (e.g. normal flora)
- airborne: surrounding air or environment (e.g. dust, water droplets)
- direct contact: equipment or hands of carers (e.g. septic techniques).

Infection is primarily diagnosed based on the presence of clinical signs and symptoms although microbial culturing is useful in selecting appropriate first line antibiotics (Kingsley, 2001). Early recognition of the signs of infection is important in limiting the effect of infection. According to Bowler *et al.* (2001) controlling the microbial load of wounds is a vital factor in minimizing infection.

Educating people about the various facets involved in wound progression, regression and the use of proper aseptic techniques, may enhance a positive outcome in wound management (Collier, 2004).

2.3.2.2 Burn wound infection

Bacterial infection is a frequent and serious infectious complication in burn recipients (Mayhall, 2003; Pandit & Gore, 1997). Burn wound infection is associated with prolonged healing and the risk of systemic infection (Palmeiri & Greenhalgh, 2002). Approximately 50 – 75% of deaths following burns are related to infections (Pandit & Gore, 1997; Schwarz, 2001). Resistance to antimicrobial agents and a generalised immune suppression are risk factors that contribute to burn wound colonization and infection (Mayhall, 2003).

In recent years burn injuries have reached epidemic proportions in the United States, mainly targeting children (Palmeiri & Greenhalgh, 2002). In the United States burn wound infections occur most frequently in children followed by the elderly (Schwarz, 2001). An Australian paediatric hospital reported treating more than 720 new cases annually, with the majority resulting from tea and coffee scalds (McCormack *et al.*, 2003). Minor burns are extremely common in children and may be attributed to its accidental nature in households (McCormack *et al.*, 2003). According to a surgical

consultant in the Nelson Mandela Metropole, burns are lethal injuries that may kill children even in the best burn care facilities, based on the degree of surface area involved in relation to their body size (Jones, 2002).

In the United States an estimated 2.5 million people sustain burns that require medical attention annually (Mayhall, 2003). Infected burn wounds increases the length of hospital stay and treatment costs (Arslan *et al.*, 1999). Burn wounds resulting in a loss of the protective skin coverage forms a favourable entry route for microbial colonization. Burn injuries facilitates microbial growth in the moist, protein rich avascular eschar that replaces the normal skin barrier (Schwarz, 2001).

Burn wounds are normally described based on the percentage involvement of body surface area (%BSA) and the depth of skin injury (first, second or third degree) (Ward & Saffle, 1995). Burns are classified skin traumas according to the depth of injury in the different skin layers (Figure 2) as:

- | | | |
|----------------------------|------------------------|---|
| ▪ Superficial injury | 1 st degree | limited to the epidermis with heat, pain and reddening e.g. sunburns. |
| ▪ Partial thickness injury | 2 nd degree | extend to dermis including extreme pain and blister formation. |
| ▪ Full thickness injury | 3 rd degree | extends to the subcutaneous layer with a leathery appearance. |

Burns are serious injuries that should not be underestimated in terms of immediate attention and management required in alleviating infectious complications. The outcome of burn injuries is multifactorial and may differ from one person to another, depending on the degree of injury and their general health status. Severe burns are complex injuries that require specialized assistance in providing a positive outcome.

Awareness of appropriate first-aid for burn injuries is important due to its accidental nature and benefits of alleviating patient discomfort. Immediate cooling of burns using cool running water for 10 – 30 minutes are beneficial in reducing pain, swelling and hospitalisation (Skinner *et al.*, 2004; McCormack *et al.*, 2003). Cooling the affected burn area as first-aid should be accompanied by keeping the rest of the

body warm to prevent hypothermia, especially in children (Skinner *et al.*, 2004; McCormack *et al.*, 2003).

Some management measures for burn wound infections include removal of necrotic tissue, rational antibiotic therapy, enhancing the immune response and providing adequate nutrition (Bagdonas *et al.*, 2003; Bowler *et al.*, 2001). Successful treatment of burn wounds relies on understanding the spectrum of common pathogens, mechanisms of resistance and infection control procedures (Kanchanapoom & Khardori, 2002). According to Collier (2004) it is important to treat the patient as a whole and not the infection alone, focussing on a holistic assessment of an individual.

2.3.2.3 Pathogens associated with burn wound infection

The nature of burn wound injury resulting in the disruption of skin integrity allows colonization of microorganisms from various origins that initiate wound infection. Different types of microorganisms may colonize and cause infection in burn wounds, resulting in a polymicrobial wound infection. According to Trengrove *et al.* (1996) there is an increase chance of failure to heal if four or more bacterial groups are present in the wound.

The burn wound is usually sterile immediately following injury, with different microorganisms colonizing the wound over time (Ugburo *et al.*, 2004; Schwarz, 2001). Aerobic and facultative pathogens such as *Staphylococcus aureus*, *P. aeruginosa* & *Streptococcus pyogenes* are primary causes of infection and delayed healing in wounds (Collier, 2004; Bowler *et al.*, 2001). The normal skin flora that includes Gram-positive *Staphylococcus* & *Streptococci* spp. tends to result in early burn wound infection. At a later stage more predominant Gram-negative bacteria associated with increased exudates and patient discomfort tend to colonize the burn wound (Palmeiri & Greenhalgh, 2002; Kanchanapoom & Khardori, 2002).

Although wound infections may result from aerobic and anaerobic microorganisms, aerobes are more commonly isolated due to the difficulty associated with the isolation and culturing of anaerobes (Bowler *et al.*, 2001). Bacteria were reported as

the most common causes of burn wound infection, followed by less common microbial causative agents e.g. yeasts, filamentous fungi and viruses (Mayhall, 2003).

Previous epidemiological studies that reported on the microbial assessment of infected burn wounds, indicated frequent isolations of the following bacteria from burn wound swabs:

- *P. aeruginosa* followed by *S. aureus* were reported as the most common microorganisms causing wound infection in burns (Ugburo *et al.*, 2004; Mayhall, 2003).
- Bagdonas *et al.* (2003) reported *S. aureus* as the most common infective agent in burn wounds and may often result in toxic shock.
- Studies and surveys on the bacteriology of burns have indicated frequent isolations of *K. pneumoniae*, *Proteus* spp., *Enterococci* spp., *Acinetobacter* spp. & *Candida albicans* to name a few (Bagdonas *et al.*, 2003; Pandit & Gore, 1997).
- Schwarz (2001) reported *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *E. feacalis* & *A. baumannii* as some of the most frequently isolated organisms from burn wound biopsies.

Microorganisms infecting wounds are associated with different characteristics and virulence factors that aid infection and patient discomfort. The multiplication of some anaerobes and aerobes in wounds, which include *Proteus* spp., *Klebsiella* spp. & *Pseudomonas* spp. produce uncomfortable situations e.g. a foul smelling odour (Kingsley, 2001). The production of potentially destructive virulence factors by pathogens may impair wound healing. More than 90% of *S. aureus* produce specific enzymes that inactivate certain antimicrobial agents, rendering the antimicrobial ineffective for treatment (Bagdonas *et al.*, 2003). The release of different bacterial toxins is associated with delayed healing and toxic shock.

It is important to limit the spread and invasion of microorganisms with their potentially destructive virulence factors that cause infection and delay healing. Controlling the microbial load in wound infections may limit the infection and enhance healing.

2.3.3 Bacteria selected for investigation

In this study Gram-positive and Gram-negative bacterial strains were included based on the frequency of isolation locally and in epidemiological studies of infected burn wounds.

Different clinical isolates of four Gram-positive bacteria including *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Streptococcus pyogenes* (group A) and *Enterococcus faecalis* were selected for *in vitro* antibacterial assays (Table 2).

S. aureus and *S. pyogenes* form part of the normal skin flora and are often the initial causative agents of burn wound infection arising endogenously from the patients' normal flora. The selection of *S. aureus* and *S. pyogenes* is based on its frequency of isolation and their ability of causing early infection in burn wounds (Schwarz, 2001; Bagdonas *et al.*, 2003).

Methicillin-resistant *S. aureus* is more virulent than *S. aureus* and has modified protective/virulence mechanisms resulting in an increased antimicrobial resistance to a variety of antimicrobials. The increased antimicrobial resistance make methicillin-resistant *S. aureus* more difficult to control and eradicate (Bagdonas *et al.*, 2003). Therefore methicillin-resistant *S. aureus* was included in the bacterial selection to investigate the usefulness of medicinal plant extracts in treating and controlling methicillin-resistant *S. aureus* infected wounds. Methicillin-resistant *S. aureus* is a widespread nosocomial pathogen and was reported to have the highest prevalence in specific clinical wards especially in the burn units (Narezkina *et al.*, 2002).

E. faecalis is an opportunistic pathogen forming part of the intestinal and faecal flora (Greenwood *et al.*, 1997). *E. faecalis* may survive in hospital environments due to its intrinsic resistance to commonly used antibiotics (Kanchanapoom & Khardori, 1999). Infections of *E. faecalis* may arise endogenously from the patient's own flora and/or through transmission of direct contact. Similar to methicillin-resistant *S. aureus* with increased antimicrobial resistance, vancomycin-resistant enterococci (VRE) infections are difficult to control and eradicate.

The four different Gram-negative bacteria selected for *in vitro* antibacterial testing in this study included *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Proteus mirabilis* and *Klebsiella pneumoniae* (Table 2).

P. aeruginosa was reported as being one of the most common bacteria causing infection in burn wounds (Ugburo *et al.*, 2004; Mayhall, 2003; Collier, 2004). *P. aeruginosa* is an opportunistic, environmental pathogen and may contaminate the floors, bed rails, sinks of hospitals, and hands of health care workers. Burn wounds infected with *P. aeruginosa* may be difficult to control and eradicate, due to multi-resistance to antimicrobial agents (Mayhall, 2003; Arslan *et al.*, 1999). The virulence factors of *P. aeruginosa*, such as protease & exotoxins, promote infection and the dissemination of infection (Table 2) (Kanchanapoom & Khardori, 1999).

The non-fermenting *A. baumannii* is an opportunistic, environmental pathogen with a high survival rate in various environmental locations that may facilitate the development of nosocomial infections. *A. baumannii* has been isolated from different locations especially in the hospital environment including pillows, washcloths, telephone handles, pasteurised milk and ventilators (Greenwood *et al.*, 1997). The ability of *A. baumannii* to develop antimicrobial resistance with extreme rapidity makes it difficult to control and eradicate using routine antibiotics (Kanchanapoom & Khardori, 2002).

Both *P. mirabilis* and *K. pneumoniae* are opportunistic pathogens that may result in nosocomial infections (Table 2). *P. mirabilis* and *K. pneumoniae* form part of the normal intestinal flora that may predispose burn wounds to infection (Greenwood *et al.*, 1997). The virulence factors of *P. mirabilis* and *K. pneumoniae* include peritrichous flagella and large mucoid capsules, respectively.

The bacteria selected for antimicrobial investigation have different virulence mechanisms influencing the outcome of a wound infection. A summary of the selected bacteria commonly associated with burn wound infections and their microbial characteristics are displayed in Table 2.

TABLE 2 Summary of the selected test bacteria and their microbial characteristics

	Microbial characteristics:	Virulence & microbial factors:	Associated infections:	References:
Gram-positive bacteria:				
<i>Staphylococcus aureus</i>	normal commensal coagulase +ve, catalase +ve	staphylococcal & membrane-damaging toxins eg. protease, hyaluronidase, coagulase etc.	skin infections, wound infection toxic shock, septicemia etc.	(Greenwood <i>et al.</i> , 1997; Nester <i>et al.</i> , 2004)
methicillin-resistant <i>Staphylococcus aureus</i>	occasional nose flora coagulase +ve, catalase +ve	penicillinase which aid resistance & toxins eg. protease, coagulase, hyaluronidase etc.	wound infection in severe burns septicemia & skin infections	(Greenwood <i>et al.</i> , 1997; Bagdonas, 2003)
<i>Streptococcus pyogenes</i>	Lancefield group A beta-hemolysis, bacitracin sensitivity & catalase -ve	streptococcal toxins & enzymes eg. hemolysin, protease, streptokinase.	wound infection, tonsillitis, impetigo & septicemia	(Greenwood <i>et al.</i> , 1997; Bowler <i>et al.</i> , 2001; Nester <i>et al.</i> , 2004)
<i>Enterococcus faecalis</i>	Lancefield group D opportunistic pathogen normal intestinal flora & aesculin bile salt +ve	widespread antimicrobial resistance eg. vancomycin resistant enterococci (VRE)	nosocomial infections, skin & wound infections, bacteremia	(Greenwood <i>et al.</i> , 1997; Kanchanapoom & Khardori, 2002)
Gram-negative bacteria:				
<i>Pseudomonas aeruginosa</i>	opportunistic pathogen environmental pathogen oxidase +ve	innate antimicrobial resistance, toxins & enzymes eg. protease & exotoxins.	nosocomial infections, burn wound infections & cystic fibrosis	(Greenwood <i>et al.</i> , 1997; Bowler <i>et al.</i> , 2001; Arslan <i>et al.</i> , 1999)
<i>Acinetobacter baumannii</i>	opportunistic pathogen environmental pathogen oxidase -ve	intrinsic resistance to many antibiotics, endotoxins	nosocomial infections, burn wound infection & bacteraemia	(Greenwood <i>et al.</i> , 1997; Kanchanapoom & Khardori, 2002)
<i>Proteus mirabilis</i>	opportunistic pathogen normal intestinal flora swarming on blood agar & indole -ve & urease +ve	peritrichous flagella & endotoxins	nosocomial infections, burn wound infection & urinary tract infections	(Greenwood <i>et al.</i> , 1997; Arslan <i>et al.</i> , 1999)
<i>Klebsiella pneumoniae</i>	opportunistic pathogen normal intestinal flora mucoid colonies on agar indole -ve & urease +ve	natural resistance to most penicillins large mucoid capsule endotoxins	nosocomial infections, burn wound infection & sepsis in surgical wounds.	(Greenwood <i>et al.</i> , 1997; Arslan <i>et al.</i> , 1999)

2.3.4 Antibiotic resistance

Opportunistic pathogens and nosocomial infections are important causes of infection in burn wounds due to the compromised skin barrier in burn injuries (Bowler *et al.*, 2001). According to the WHO antibiotic resistant bacteria are responsible for up to 60% of hospital-acquired infections in the United States (World Health Report: press release, <http://www.who.int/whr/1996/press1.htm>). Bagdonas *et al.* (2003) reported that resistance is site depended, with the burns and trauma departments reported as some of the most common sites for the emergence of resistance. Antibiotic resistance is considered a global health concern and has been called one of the world's most pressing public health problems (<http://www.cdc.gov/drugresistance/community/>).

Some of the bacteria are resistant to as many as ten different drugs, raising the concern of a post-antibiotic era (<http://www.who.int/whr/1996/press1.htm>). Current trends suggest that no effective therapies will be available for treating some diseases within the next ten years (<http://www.who.int/mediacentre/factsheet/fs194/en/>). The rates of some communicable diseases have started to increase again as a result of the rise in antibiotic resistance (Levy, 1998).

Various facets contribute to the occurrence and spread of antimicrobial resistance. The uncontrolled and inappropriate use of antibiotics today, may reduce future effectiveness of the antibiotics. For example individuals chronically medicating acne with antibiotics in a household, raise the concentration of antibiotic resistant bacteria on the skin of family members (Levy, 1998). Antimicrobial soaps and detergents as well as the agricultural use of antibiotics as growth factors, increase the pressure on wild bacteria to evolve resistance (Aleksun & Levy, 2001; Berkowitz, 1995). Overcrowded and unhygienic conditions as well as international travelling and trading facilitate the spread of antibiotic resistance (<http://www.who.int/whr/1996/press1.htm>).

The increasing misuse of antibiotics has led to an international public health nightmare, with increasing bacterial resistance to many antibiotics that once readily cured bacterial diseases (Levy, 1998). With each passing decade bacteria that defy not only single but also multiple antibiotics have become increasingly common and

extremely difficult to control. Resistant nosocomial infections are expensive to control and eradicate (<http://www.who.int/mediacentre/factsheet/fs194/en/>).

Antibiotic resistance in bacteria results from the acquirement of genes conferring resistance and the use of these acquired mechanisms for expressing resistance to antimicrobial agents. Antibiotics inactivate defenceless bacteria, resulting in the selection and survival of the fittest, antibiotic resistant bacteria (Lewis, 1995). Resistant bacteria have various mechanisms to disable the harmful actions of certain antibiotics, ensuring bacterial survival, such as:

- production of enzymes that destroy the active antibiotic
- changing cell wall permeability to antibiotics
- rapid effluence/discharge of antibiotics from the interior of the bacteria and
- developing structural alteration in the attachment site for antibiotics (Lewis, 1995; Berkowitz, 1995).

The search for new effective antimicrobial agents may alleviate the difficulties associated with patient outcome and treatment of antibiotic resistant infections. The investigation and discovery of novel effective antimicrobial agents should be accompanied with an appreciation and rational use of current antibiotics. Scientific investigation of traditionally used medicinal plants for antimicrobial properties may serve as effective agents for the treatment of antibiotic resistant infections. Eloff (1998) suggested that antimicrobial agents originating from plants might use a different mechanism to inhibit microorganisms and resistant pathogens.

Implementation of simple infection control practices such as hand washing, use of protective clothing and aseptic techniques may limit the spread of resistant microbes, especially in hospitals. The WHO launched a global strategy in 2001 for combating antimicrobial resistance, aimed at slowing the emergence and reducing the spread of resistance (<http://www.who.int/mediacentre/factsheet/fs194/en/>). Antibiotic resistance is inevitable, but measures such as infection control, development of new antimicrobial agents and rational use of effective antimicrobial agents may slow resistance (Lewis, 1995).

CHAPTER 3

METHODOLOGICAL JUSTIFICATION

This study is aimed at the investigation of different extractions and traditional preparations of medicinal plants for *in vitro* antibacterial activity and scientifically justifying certain traditional uses of these plants. The bacteria used in this study are clinical isolates from burn wound patients obtained from the National Health Laboratory Services (NHLS) in Port Elizabeth.

The selection of methods and techniques for investigating the *in vitro* antibacterial activity of medicinal plants can be a challenging task when faced with the various methods employed in literature. The different requirements of the selected bacteria and the traditional uses of the medicinal plants formed the basis for selecting the methods used in this research.

3.1 Medicinal plants

The medicinal plants (*Bulbine frutescens*, *Leonotis leonurus*, *Melianthus major*, *Zantedeschia aethiopica*) were selected based on their traditional therapeutic uses as described in literature. The selected medicinal plants are traditionally used in the Eastern Cape region for treating burns and skin conditions (Van Wyk *et al.*, 1997; Van Wyk & Gericke, 2000). Although different parts of a plant may show varying antibacterial activity (Essawi & Srour, 2000), leaves were reported as the most frequently used plant part for treating wounds in the Eastern Cape Province, South Africa (Grierson & Afolayan, 1999b).

Fresh plant leaves that were easily accessible on a regular basis from the site of collection were used for extracting different active compounds. The plant leaves were crushed into fine leaf particles in order to obtain a large surface area for solvent extraction. Although the interfering water content of fresh plants may pose problems when there is a delay between collection and processing (George *et al.*

2001), the traditional method of preparation of the selected plants employ the use of fresh leaves (Van Wyk *et al.*, 1997). According to George *et al.* (2001), it is essential that traditional methods of processing plants be taken into account when standardizing procedures for phytochemical investigation.

3.2 Extraction of medicinal plants

An adapted quantitative extraction method was performed in triplicate (Eloff, 1998b). The plant material was extracted with three different extraction solvents in order to obtain a dried extract residue. The solvent-free dried extract residues were resuspended in dimethylsulphoxide (DMSO) for subsequent analysis.

The antibacterial activity varies with the extraction solvent. It is therefore essential to select the most appropriate solvent for extracting antibacterial compounds (Lin *et al.*, 1999; Vlachos *et al.*, 1996). A specific extractant (solvent) may extract different antimicrobial compounds from plants with different chemical profiles. Three different extraction solvents namely methanol, acetone and distilled water (aqueous) were selected based on a review of literature for extracting different constituents from the plants.

Methanol followed by acetone was reported as the most effective solvents for extracting antibacterial compounds from plants (Vlachos *et al.*, 1996). Studies revealed that besides the efficacy of methanol as an antibacterial extractant, it gave the most consistent antibacterial activity whereas acetone extracted a complex mixture of different components (Lin *et al.*, 1999; Martini & Eloff, 1998). Aqueous extracts of plants was included due to the reported popularity for use as solvents in preparing remedies for traditional medicinal purposes (Brantner & Grein, 1994). In addition to the three different solvent extractions, traditional preparations (decoctions & infusion) of the medicinal plants were subjected to *in vitro* antibacterial analysis.

3.3 Assays for antibacterial analysis

An adapted serial microtitre plate assay using 96-well microtitre plates was used for assessing the *in vitro* antibacterial activity of medicinal plants against the selected

bacterial strains. The microtitre plate assay using INT (*p*-iodonitrotetrazolium) as a bacterial growth indicator gave reproducible results and allowed determination of the MIC of each plant extract (Eloff, 1998a & Eloff, 1998b).

Initially the different plant extracts and traditional preparations were screened with the bacterial strains in triplicate using the microtitre plate assay.

Certain bacteria, *S. pyogenes*, *E. faecalis* & *P. mirabilis*, were not compatible with the microtitre plate assay using distilled water as the diluent and a microtitre plate reader for analysis. Therefore an adapted agar dilution plate assay (Boswell *et al.*, 2001; Andrews, 2004) was used as an alternative method for determining the antibacterial activity and MIC's of the medicinal plant extracts with these bacteria.

The agar dilution plate assay incorporated the use of different dilutions of the plant extract mixed into melted agar (Kerr *et al.*, 1999; Andrews, 2004).

The equipment and techniques employed for investigating medicinal plant extracts may play a role in the accuracy of reporting and comparing the results in different studies. A schematic representation of the different steps performed in this study for assessing the *in vitro* antibacterial activity of the selected medicinal plants is showed in Figure 3.

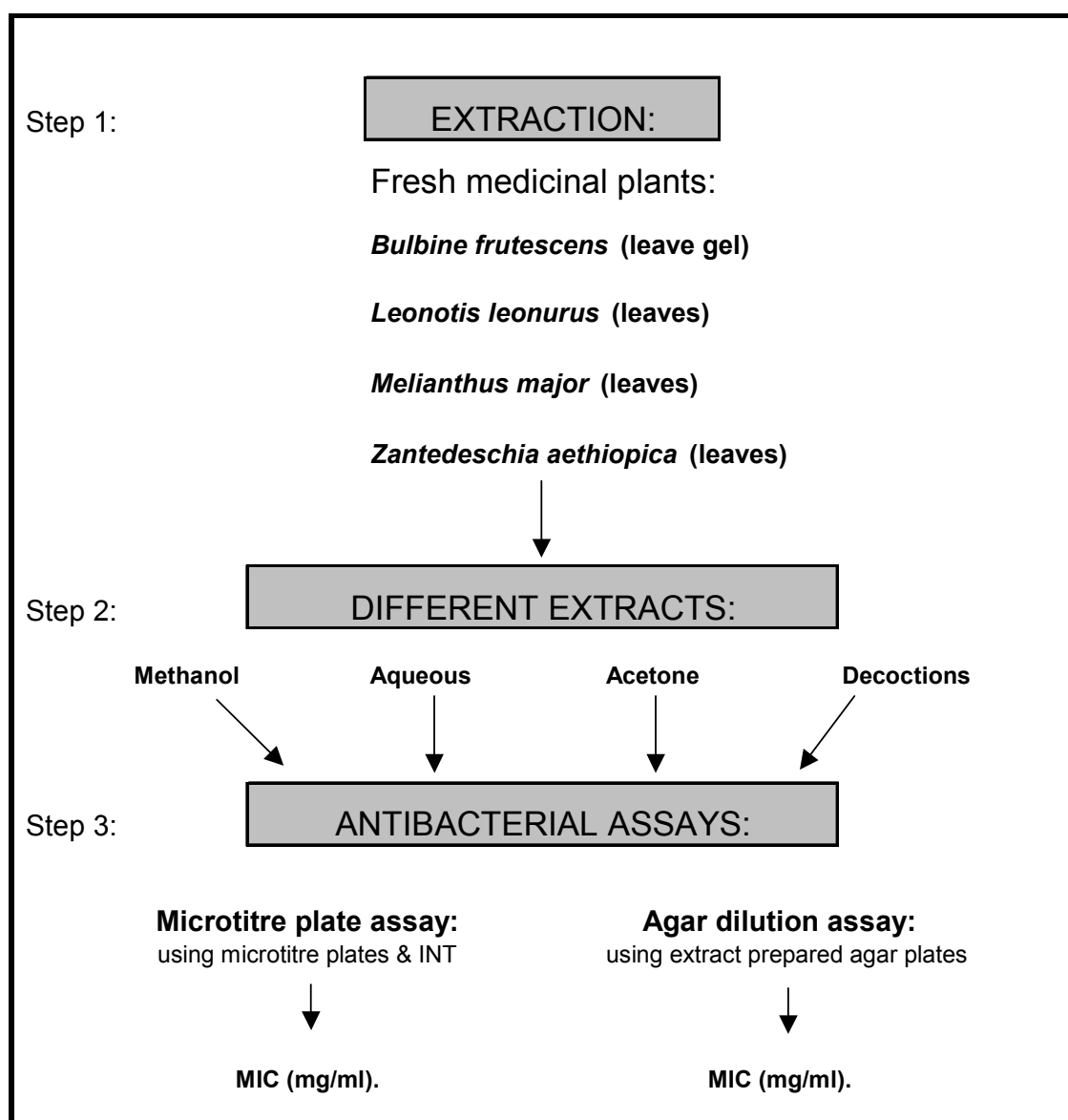


FIGURE 3 Schematic representation of the different steps performed for assessing the antibacterial activity of the medicinal plants

CHAPTER 4

MATERIALS AND METHODS

4.1 Plant selection and preparation

The selection of all four medicinal plants was based on their reported traditional uses in treating wound infections especially in the Eastern Cape region. All the plants were easily accessible and readily available for collection from the UPE (University of Port Elizabeth) gardens. The accessibility of the plants allowed immediate processing of fresh plant parts, prior to extraction. The plants were identified and authenticated by E. Campbell in the Botany department of the University of Port Elizabeth. The leaves were cleaned by washing with distilled water and sorted to eliminate any old, damaged leaves.

The leaves of the three plants (*Leonotis leonurus*, *Melianthus major* and *Zantedeschia aethiopica*) were crushed into fine plant particles and sap, using a porcelain pestle and mortar. The clear inner gel of the succulent *Bulbine frutescens* leaves was obtained, by removing most of the outer green layer with a sterile surgical blade. The thickness of the succulent *Bulbine frutescens* leaves varied occasionally with plant collection. Difficulty was occasionally experienced in obtaining the inner gel from the thinner, less succulent leaves. The freshly crushed plant leaves and gel was immediately extracted with the different solvents.

4.2 Plant extract and traditional preparations

4.2.1 Plant extraction

An extraction method adapted from Eloff (2000) was employed using approximately 5g of freshly processed plant leaves for the three plants and 2.5g for the *Bulbine* leave gel. A schematic representation of the extraction procedure illustrates the

different steps performed to obtain the plant extract supernatants (Table 3). The extraction procedure was performed as follows with the fresh plant material:

DAY 1 – Plant extraction

- Methanol, acetone and sterile distilled water (aqueous) were chosen as the extraction solvents (extractants) for extracting different compounds from the plant material, respectively.
- Weighed plant material and 5ml of each solvent were placed in screw cap tubes, respectively and shaken vigorously for 5 min (Table 3).
- Centrifugation of different extracts in tubes for 5 min separated the supernatant from the plant material for each extract.
- Supernatant of each plant extract was transferred into pre-weighed beakers (Table 3).
- Procedure was repeated twice more for re-extracting the remaining plant material with additions of 5ml solvent, respectively.
- The pre-weighed beakers containing the plant extract supernatants were allowed to dry completely to obtain a solvent-free dried extract residue.
- Methanol and acetone plant extract supernatants were subjected to overnight drying under airflow in a fume cupboard.
- Incomplete drying of the aqueous plant extract supernatants prompted alternative drying at 37°C overnight.

DAY 2 – Plant extraction

- Dried beakers were reweighed and calculated extract residues resuspended in 0.5ml DMSO [Merck].
- Plant extract concentrations varied between the different plants and solvents used for extraction.
- Prior to assaying, a 1.25% extract solution was made using the stock DMSO extracts and sterile distilled water.

TABLE 3 Schematic representation of the extraction procedure

(i) fresh plant material



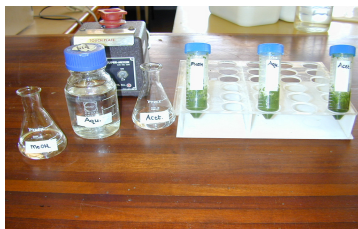
(ii) crushing fresh plant material



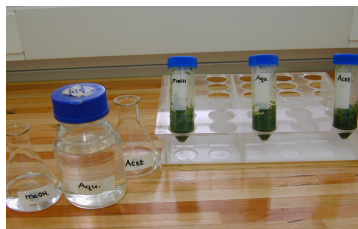
(iii) weighing plant material



(iv) plant material in tube



(v) addition of 5ml solvents



(vi) vigorous shaking – 5min



(vii) centrifugated tubes (3000rpm)



(viii) supernatants to weighed beakers



(ix) (repeat steps v – viii) x2



(x) allow overnight drying of collected supernatants to obtain dried extract residues

4.2.2 Traditional preparations

Besides processing the plants with different solvents for extraction, the traditional method of preparation for each plant was also included for analysis (Van Wyk *et al.*, 1997; Grierson & Afolayan, 1999b).

- Decoctions were made for *Leonotis leonurus*, *Melianthus major* and *Zantedeschia aethiopica*, by combining 5g leaves and 100ml distilled water in a covered glass flask boiling for 5 minutes.
- An infusion for *Bulbine frutescens* was made by pouring 100ml boiled distilled water onto 5g crushed leave gel leaving it to stand covered for 5 minutes.

4.3 Bacteria and growth conditions

Seventy-three bacterial strains isolated from burn wounds were collected in semi-solid agar at the NHLS, Port Elizabeth. Antibiotic sensitivity patterns were obtained for each of the bacteria. Semi-solid agar was stored at room temperature and sub-cultured when required.

TABLE 4 The number of bacterial strains tested for antibacterial activity

Selected bacteria:	Number of bacterial strains tested	
	Total screened:	MIC determination:
Gram-positive:		
▪ <i>Staphylococcus aureus</i>	7	4
▪ Methicillin-resistant <i>S. aureus</i>	8	4
▪ <i>Streptococcus pyogenes</i>	6	5
▪ <i>Enterococcus faecalis</i>	11	5
Gram-negative:		
▪ <i>Pseudomonas aeruginosa</i>	5	3
▪ <i>Acinetobacter baumannii</i>	13	5
▪ <i>Klebsiella pneumoniae</i>	8	5
▪ <i>Proteus mirabilis</i>	15	5
Total bacterial strains tested	73	36

The Gram-positive bacteria were sub-cultured onto blood agar plates (BA plates) obtained from NHLS and the Gram-negative bacteria were sub-cultured onto MacConkey agar (Biolab) plates, followed by overnight incubation at 37°C.

- Growth curves were performed on all test bacteria to obtain the desired optical density for a bacterial count of $10^6 - 10^8$ cfu/ml.
- Bacterial broth densities were read with a spectrophotometer (GBC UV/VIS 911A) at 600nm.

4.4 Antibacterial assays

4.4.1 Microtitre plate assay

An adapted microtitre plate assay was used to assess the antibacterial activity of plant extracts against different bacterial strains (Eloff, 1998a & Eloff, 1998b). Microbial growth or inhibition of growth was measured in round-bottomed 96-well microtitre plates with the addition of the bacterial growth indicator, *p*-iodonitrotetrazolium (INT) salts. INT (Sigma) is a growth indicator serving as an electron acceptor that is reduced from a colourless compound to a red coloured formazan product by biologically active cells, within 10 - 60 minutes (Eloff, 1998b; Gabrielson *et al.*, 2002).

The addition of INT allowed easy and reproducible measurements of bacterial growth with a microtitre plate spectrophotometer (Gabrielson *et al.*, 2002). The microtitre plate assay is quick, sensitive and gave reproducible results with Gram-positive and Gram-negative bacteria. The method required very small amounts of the extract for assaying and allowed for determination of the minimal inhibitory concentration (MIC) of plant extracts using a microtitre plate spectrophotometer (Devienne & Raddi, 2002).

DAY 1 – Microtitre plate assay

Microtitre plates contained equal volumes (50 µl) of bacterial suspension, plant extracts and bacterial growth indicator (INT) (Figure 5).

- Row A and B of the microtitre plates were filled with 50 µl plant extract (1.25%) in the top two rows.

- Twofold serial dilutions were made from row (B) to row (G) to obtain dilutions ranging from 1:2 to 1:64.
- 50 µl bacterial broth suspensions ($10^6 - 10^8$ cfu/ml) were then added to each of the microtitre plate wells.
- Bacterial growth control and sterility controls were included.
- Bacterial control well contained only water and bacterial suspension.
- All solvents including extracts, traditional preparations, diluents, growth medium were subjected to sterility assessment in microtitre plates and cultured on blood-agar plates.
- All extracts and traditional preparations (decoctions & infusions) were tested in triplicate in the microtitre plates and test performed three times.
- Covered microtitre plates placed in a closed, moist container were incubated overnight shaking (140 rpm) at 37°C.

		Columns (1-3)	Columns (4-6)	Columns (7-9)	Columns (10-12)
		Triplicate	Triplicate	Triplicate	Sterility Controls
Rows	A	Undiluted extract			
	B	1:2 extract dilution			
	C	1:4 extract dilution			
	D	1:8 extract dilution			
	E	1:16 extract dilution			
	F	1:32 extract dilution			
	G	1:64 extract dilution			
	H	ORGANISM CONTROLS			

Microtitre plates contained 50 µl of:

- Distilled H₂O
- Extract
- Bacterial broth
- INT solution

FIGURE 4 Layout of the 96-well microtitre plate: dimensions and test suspensions

DAY 2 – Reading of microtitre plates

- First reading - turbidity reading of microtitre plates read at 550 nm with the microtitre plate reader (ELX 800 – Biotek) while ensuring no moisture or fingerprints on microtitre plate reading surface.

- 50 µl of 0.2 mg/ml INT [Sigma] solution using phosphate buffered saline (PBS) at pH 7.5 as the diluent for INT, were added to each of the microtitre plate wells.
- Microtitre plates were incubated for a further 45 – 60 min on a shaker (140 rpm) in an incubator at 37°C.
- The second and final reading of the microtitre plates at 550 nm with the microtitre plate reader, recorded the absorbance of coloured formazan products in the wells.
- The two readings (turbidity and INT reading) formed the basis for calculating the percentage bacterial growth/inhibition in relation to organism control wells (Figure 5).
- The turbidity readings were subtracted from the INT readings to obtain the subtracted values that were used in the calculation as displayed below (Figure 5).

The subtracted values of the test wells were divided by the respective organism control wells to obtain the percentage bacterial growth in each well. The percentage inhibition for each well was obtained by subtracting the percentage bacterial growth from 100.

$$\frac{[\text{INT reading}_{(\text{test wells})} - \text{Turbidity reading}_{(\text{test wells})}]}{[\text{INT reading}_{(\text{bacteria control})} - \text{Turbidity reading}_{(\text{bacteria control})}]} \times 100 = \% \text{ Bacterial growth}$$

$$\therefore 100 - \% \text{ bacterial growth} = \% \text{ Bacterial inhibition}$$

FIGURE 5 Representation of the calculation performed to obtain the bacterial inhibition

The absorbance of the red-coloured formazan product of bacterial suspensions should be detectable with the spectrophotometer for analysis and interpretation of the antibacterial activity as percentages.

Enterococcus faecalis, *Streptococcus pyogenes* & *Proteus mirabilis* did not reduce the growth indicator, INT (Figure 6). Therefore the bacterial growth of these bacteria could not be detected spectrophotometrically. A previous study done by Eloff (1998a) reported that *E. faecalis* reacted slower than other microorganisms using the

microtitre plate assay. Following prolonged incubation periods (90 – 120 minutes) of the three bacteria only slight or no colour change were visible in comparison with the other bacteria (Figure 6). Based on these findings it was decided to substitute the distilled water diluent in the microtitre plate assay with an enriched medium, Mueller Hinton (MH) broth. Although using MH broth as an alternative diluent, the absorbance values of these bacteria obtained with the microtitre plate reader still gave high rates of inconsistency in comparison to the other test bacteria.

The three bacteria were not compatible with the microtitre plate assay and its subsequent spectrophotometric microtitre plate analysis in this study. The other bacteria *S. aureus*, *P. aeruginosa* and *K. pneumoniae* were successfully reduced by INT tested on the same microtitre plates (Figure 6).

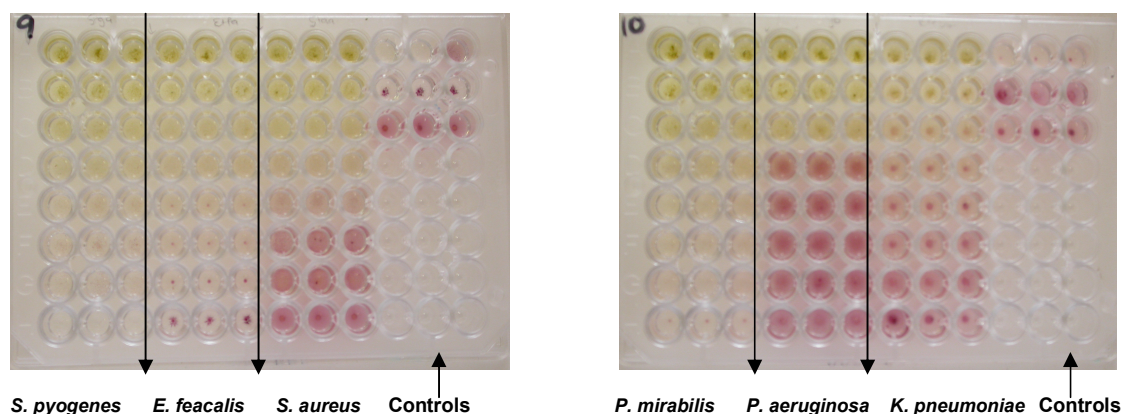


FIGURE 6 Lack of INT reductions by the three bacteria

4.4.2 Agar dilution assay

The strains of the three bacteria, *E. faecalis*, *S. pyogenes* & *P. mirabilis*, that produced inconsistent absorbance readings with the microtitre plate assay were tested with an adapted agar dilution assay. The agar dilution assay allowed for the determination of the MIC's on agar plates prepared with plant extracts at different concentrations (Boswell *et al.*, 2001; Andrews, 2004).

The agar plates containing plant extracts and a 36-pin multipoint inoculator (Mast diagnostics, UK) device, served as the basis for antibacterial activity testing of the plant extracts. The agar dilution assay using a multipoint inoculator, worked well in

previous studies with susceptibility testing of *Proteus* species (Stratchounski *et al.*, 1999) as well as *Enterococcus* & *Streptococcus* species (Andrews *et al.*, 1999).

Screening was done for all the plant extracts at different concentrations against all the bacterial strains. The final plant extract concentrations in the agar plates ranged between 0.5 mg/ml and 20 mg/ml.

4.4.2.1 Agar plate preparation

- The extraction procedure and the volume of the redissolving solvent (DMSO) were adjusted to obtain a uniform stock solution concentration for each plant extract.
- Serial dilutions of the stock solution for each extract and traditional preparation were made to a constant volume of 500 µl with sterile distilled water to facilitate proper mixing of the extract and agar in the petri dishes.
- The 500 µl plant extract dilutions were carefully mixed into approximately 20 ml diagnostic sensitivity test (DST) agar to obtain the final agar plate concentrations ranging between 0.5 and 20 mg/ml.
- Proper mixing of the extract with the sensitivity agar for a uniform extract distribution was ensured and plates were allowed to set on a level dry surface.
- Blood supplemented DST agar were used to facilitate the growth of *E. faecalis* & *S. pyogenes*.
- Supplementation of *p*-nitrophenyl glycerol (PNPG) to the diagnostic sensitivity agar prevented the swarming of *P. mirabilis* on the test plates, improving the reading of the plates for analysis (Figure 7).
- Growth control plates were included for both Gram-positive and Gram-negative test bacteria on BA plates and MacConkey agar (Biolab) plates, respectively.
- Agar plates supplemented with the redissolving solvent (DMSO) at a percentage representative of the test extract plates were included as controls.



Plates supplemented with PNPG



No PNPG added

FIGURE 7 Reduced swarming of *P. mirabilis* on DST agar plates supplemented with PNPG

- A standard antibiotic plate, cotrimoxazole and control organisms (*S. aureus*, *E. coli*, *P. aeruginosa*) were included as controls.
- All the prepared plates were used on the day of preparation or stored refrigerated (4°C) for use within one week of preparation.

4.4.2.2 Agar plate inoculation

- An inoculum for each bacterial strain was prepared by mixing colonies from plate cultures (≤ 48 hrs) with 5 ml sterile distilled water and standardized to 0.5 McFarland density.
- The test inocula were transferred into the sterile inoculum wells of the multipoint-inoculating device (Mast diagnostics, UK), starting with the three control bacterial suspensions followed by the test bacterial suspensions.
- The extract plates were inoculated using the sterile inoculator pins of the multipoint inoculator (Mast diagnostics) to transfer 1 - 2 μ l of different inocula onto agar plates containing extracts.
- After inoculation plates were allowed to dry on the bench, before overnight incubation at 37 °C.
- Bacterial inhibition assessment was based on the analysis of growth on control plates and the absence or presence of growth on the test extract plates.

4.4.3 Standard agar plate count technique

Calculation of the results in the microtitre plate assay indicated that the plant extract might be stimulating the bacterial growth as values higher than 100% were obtained. Colony counts of the controls and specific bacterial strains were compared to assess if the bacterial strains with percentages of more than 100% were stimulated at the specific plant extract concentration.

The standard agar plate count technique (Reynolds, 2004) was used to verify the relative growth/inhibition percentages of the bacteria in specific microtitre plate extract suspensions. The aim of this method was to visibly display the relative growth/inhibition percentages of test bacteria in specific plant extracts in relation to controls, by means of comparative colony counts.

- After overnight incubation of microtitre plates, before the addition of INT, ten-fold serial dilutions were made from the microtitre plate bacterial suspensions.
- The bacteria controls containing no plant extracts were also serially diluted to determine the cfu/ml.
- Aliquots (10 µl) of selected dilutions of the microtitre plate bacterial suspensions were spread onto agar plates and incubated at 37 °C overnight.

CHAPTER 5

RESULTS

Different solvent extracts of four medicinal plants (*Bulbine frutescens*, *Leonotis leonurus*, *Melianthus major* & *Zantedeschia aethiopica*) were tested for antibacterial activity against Gram-positive and Gram-negative bacterial strains using the microtitre plate assay and agar dilution assay.

5.1 Microtitre plate assay

5.1.1 Undiluted plant extract concentrations

Table 5 is a representation of the average concentration of plant material extracted from the different extraction solvents, prior to the microtitre plate assay. The freshly processed plant material (*Bulbine frutescens*, *Leonotis leonurus*, *Melianthus major* & *Zantedeschia aethiopica*) was extracted thrice with each extraction solvent (methanol, aqueous & acetone) respectively. The leave gel of *B. frutescens* (2.5g) extracted with the different solvents was less than the amount of leave plant material extracted for the other three plants (5g).

The acetone solvent yielded the highest amount of plant material extracted from all the different medicinal plants. Extraction with distilled water (aqueous) for all the different medicinal plants produced the lowest extract concentrations in comparison to using methanol and acetone, except for *Leonotis leonurus*. The aqueous extract concentration of *L. leonurus* was higher than its counter methanol and acetone extractions for *L. leonurus* (Table 5).

Methanol extracted similar amounts of plant material as the acetone solvent from the different plants and produced slightly higher extract concentrations for *Bulbine frutescens* and *Zantedeschia aethiopica* (Table 5). There was only a slight margin of

difference in the average extract concentrations (0.06 mg/ml) (Table 5) between acetone and methanol as extraction solvents.

The aqueous extract of *B. frutescens*, 0.43 mg/ml was the lowest plant extract concentration in comparison to the other plant extracts. The acetone extract of *M. major*, 2.17 mg/ml was the highest plant extract concentration (Fig. 8 and Table 5).

Melianthus major followed by *Leonotis leonurus* were the plants that produced the highest average plant extract concentrations with the different solvents. *Zantedeschia aethiopica* followed by *Bulbine frutescens* had lower plant extract concentration yields following extraction with the different solvents (Fig. 8 and Table 5).

Following resuspension of the dried extract residues with DMSO, a 1.25% solution was prepared from the dark coloured resuspended extracts on the day of the microtitre plate assay. The plant extract solutions were represented in the initial, undiluted microtitre plate wells following addition of equal amounts bacterial broth. The less intensely coloured plant extract solutions permitted the visible and spectrophotometric observation of bacterial growth indicated by INT in the microtitre plates.

TABLE 5 Average concentration of the undiluted plant extracts in the microtitre plate wells

Medicinal plants:	Average concentrations (mg/ml) of plant extracts in undiluted microtitre plate wells:		
	Methanol	Aqueous	Acetone
<i>Bulbine frutescens</i>	0.51	0.43	0.51
<i>Leonotis leonurus</i>	1.05	1.19	1.11
<i>Melianthus major</i>	1.89	1.66	2.17
<i>Zantedeschia aethiopica</i>	1.09	0.62	1.00
Solvent average:	1.14	0.98	1.20

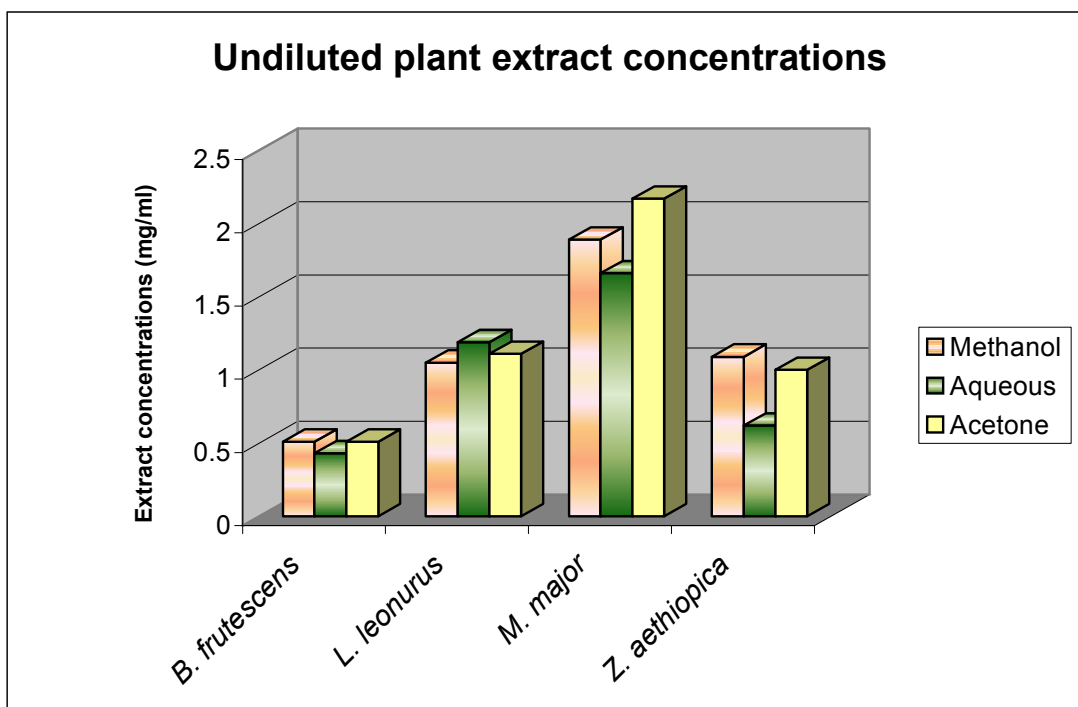


FIGURE 8 Plant extract concentrations in the undiluted microtitre plate wells

5.1.2 Visible representation of MIC's in the microtitre plates

The bacterial growth in the microtitre plates were indicated and detected with the use of a bacterial growth indicator, INT. Spectrophotometric readings of the microtitre plates before and after the addition of INT facilitated the analysis of antibacterial activity of the plant extracts.

The INT indicates the presence of viable bacteria in the microtitre plate suspensions by acting as an electron acceptor for the bacteria to form a red-coloured formazan product (Figure 9). After INT addition and incubation of the microtitre plates the presence of antibacterial activity could be assessed with the naked eye (Figure 9). Antibacterial activity was indicated by the absence of the red-coloured formazan product in microtitre plate suspensions (Figure 9).

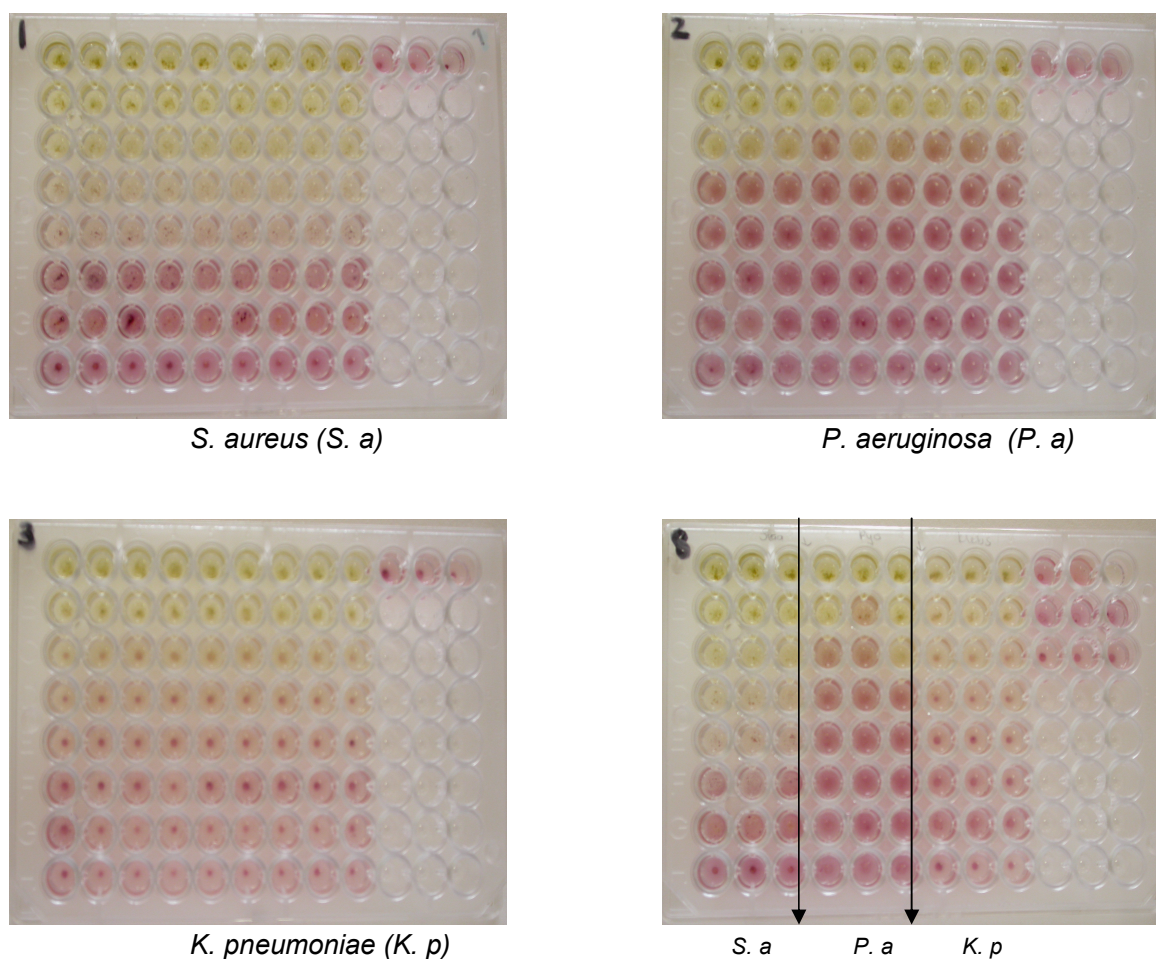


FIGURE 9 Representations of MIC's for acetone extracts of *M. major* in the microtitre plates

5.1.3 Antibacterial activity screening and MIC determination

A total of 73 bacterial strains were tested for antibacterial activity using the microtitre plate assay and the agar dilution assay. Thirty-two of the bacterial strains were Gram-positive and 41 strains were Gram-negative. All 73 bacterial strains were initially screened for antibacterial activity with the respective antibacterial assays before the determination of the MIC's of the plant extracts.

Fifty-eight bacterial strains were screened with the microtitre plate assay and 15 bacterial strains were screened with the agar dilution assay. Table 6 indicates the

number of bacterial strains of which the MIC's were obtained for the plant extracts using either the microtitre plate or the agar dilution assays.

The microtitre plate assay gave reproducible results when testing the MIC's of the medicinal plant extracts against a total of 21 bacterial strains of *S. aureus* (4 strains), MRSA (4 strains), *P. aeruginosa* (3 strains), *A. baumannii* (5 strains) & *K. pneumoniae* (5 strains) using the microtitre plate assay.

Antibacterial activity screening of plant extracts were initially performed against bacterial strains of *S. pyogenes* [no. 6], *E. faecalis* [no.'s 6 – 11] and *P. mirabilis* [no.'s 6 – 15]. The results of these bacteria were inconsistent and not compatible with the spectrophotometric method of analysis, therefore the agar dilution assay was used for these bacteria.

In the agar dilution assay the MIC's of the plant extracts were tested against 15 bacterial strains of *S. pyogenes* (5 strains), *E. faecalis* (5 strains) and *P. mirabilis* (5 strains) (Table 6).

TABLE 6 Antibacterial activity screening of the medicinal plants with different bacterial strains

Bacterial strains		Microtitre plate assay		Agar dilution assay	
	Strain No.	Screened	MIC determination	Screened	MIC determination
Gram + ve: <i>S. aureus</i>	[1]	+	+	NT	NT
	[2]	+	+	NT	NT
	[3]	+	+	NT	NT
	[4]	+	+	NT	NT
	[5]	+	NT	NT	NT
	[6]	+	NT	NT	NT
	[7]	+	NT	NT	NT
MRSA	[1]	+	+	NT	NT
	[2]	+	+	NT	NT
	[3]	+	+	NT	NT
	[4]	+	+	NT	NT
	[5]	+	NT	NT	NT
	[6]	+	NT	NT	NT
	[7]	+	NT	NT	NT
	[8]	+	NT	NT	NT
<i>E. feacalis</i>	[1]	NT	NT	+	+
	[2]	NT	NT	+	+
	[3]	NT	NT	+	+
	[4]	NT	NT	+	+
	[5]	NT	NT	+	+
	[6]	+	NT	NT	NT
	[7]	+	NT	NT	NT
	[8]	+	NT	NT	NT
	[9]	+	NT	NT	NT
	[10]	+	NT	NT	NT
	[11]	+	NT	NT	NT

TABLE 6 *Continues:*

<i>S. pyogenes</i>	[1]	NT	NT	+	+
	[2]	NT	NT	+	+
	[3]	NT	NT	+	+
	[4]	NT	NT	+	+
	[5]	NT	NT	+	+
	[6]	+	NT	NT	NT
Gram - ve:					
<i>P. aeruginosa</i>	[1]	+	+	NT	NT
	[2]	+	+	NT	NT
	[3]	+	+	NT	NT
	[4]	+	NT	NT	NT
	[5]	+	NT	NT	NT
<i>A. baumannii</i>	[1]	+	+	NT	NT
	[2]	+	+	NT	NT
	[3]	+	+	NT	NT
	[4]	+	+	NT	NT
	[5]	+	+	NT	NT
	[6]	+	NT	NT	NT
	[7]	+	NT	NT	NT
	[8]	+	NT	NT	NT
	[9]	+	NT	NT	NT
	[10]	+	NT	NT	NT
	[11]	+	NT	NT	NT
	[12]	+	NT	NT	NT
	[13]	+	NT	NT	NT
<i>K. pneumoniae</i>	[1]	+	+	NT	NT
	[2]	+	+	NT	NT
	[3]	+	+	NT	NT
	[4]	+	+	NT	NT
	[5]	+	+	NT	NT
	[6]	+	NT	NT	NT
	[7]	+	NT	NT	NT
	[8]	+	NT	NT	NT

TABLE 6 *Continues:*

<i>P. mirabilis</i>	[1]	NT	NT	+	+
	[2]	NT	NT	+	+
	[3]	NT	NT	+	+
	[4]	NT	NT	+	+
	[5]	NT	NT	+	+
	[6]	+	NT	NT	NT
	[7]	+	NT	NT	NT
	[8]	+	NT	NT	NT
	[9]	+	NT	NT	NT
	[10]	+	NT	NT	NT
	[11]	+	NT	NT	NT
	[12]	+	NT	NT	NT
	[13]	+	NT	NT	NT
	[14]	+	NT	NT	NT
	[15]	+	NT	NT	NT

(NT): Not tested, (+): Tested

5.1.4 Results of antibacterial activity screening of plant extracts

A total of 58 bacterial strains of different bacteria (*S. aureus*, MRSA, *P. aeruginosa*, *A. baumannii* & *K. pneumoniae*, *E. feacalis*, *S. pyogenes*, *P. mirabilis*) were screened for antibacterial activity using the microtitre plate assay (Table 6). The antibacterial activity of the plant extracts in the microtitre plate assay was only reported for 41 bacterial strains of *S. aureus*, MRSA, *P. aeruginosa*, *A. baumannii* & *K. pneumoniae*. The antibacterial activity of the plants against the remaining 17 bacterial strains of *S. pyogenes* [strain no. 6] *E. feacalis* strains [no.'s 6 – 11] and *P. mirabilis* strains [no.'s 6 – 15] were not reported (Table 6 & 7). The bacterial strains of these bacteria were not compatible with the microtitre plate assay and gave inconsistent results.

The screening served as an indication of the selection of bacterial strains that displayed antibacterial activity for further testing to determine the MIC's of plant extracts. Three bacterial strains *S. aureus* [strain no. 5], *P. aeruginosa* [strain no. 5] and *A. baumannii* [strain no. 13] were not viable for testing with the specific plant extracts.

The different extracts of *B. frutescens* displayed inhibition of four [no.'s 1, 3, 4 & 7] of the seven *S. aureus* strains. None of the eight bacterial strains of MRSA displayed any inhibition with the extracts of *B. frutescens* (Table 7).

The extracts of *B. frutescens* displayed antibacterial activity against three [no.'s 3, 4 & 5] of the five *P. aeruginosa* bacterial strains respectively (Table 7).

Six [no.'s 1, 4, 6, 8, 10 & 11] *A. baumannii* strains were inhibited by the methanol extract of *B. frutescens* while two [no.'s 1 & 4] and four [no.'s 1, 4, 6 & 8] bacterial strains were inhibited by the aqueous and acetone extracts respectively. All eight of the *K. pneumoniae* strains [no.'s 1 – 8] displayed inhibition with the methanol extract of *B. frutescens*. The aqueous and acetone extracts of *B. frutescens* inhibited all the *K. pneumoniae* strains except strain [no. 5] and strain [no. 2], respectively.

Leonotis leonurus extracts inhibited all the *S. aureus* strains except strain [no. 3] (methanol extract) and strain [no. 5] (aqueous extract). All MRSA strains were inhibited by the extracts of *L. leonurus* except strain [no. 4] (methanol extract) and strain [no. 7] (acetone extract) (Table 7).

The methanol and acetone extracts of *L. leonurus* inhibited all five strains of *P. aeruginosa* while the aqueous extract inhibited three bacterial strains [no.'s 2, 3 & 5]. All except two [no.'s 1 & 3] of the thirteen *A. baumannii* strains were inhibited by the methanol extracts of *L. leonurus*. Nine *A. baumannii* strains [no.'s 2, 4 & 7 – 13] were inhibited by the acetone extract and only four strains [no.'s 2 & 11 – 13] by the aqueous extract of *L. leonurus*. The aqueous extracts of *L. leonurus* inhibited all eight strains of *K. pneumoniae*. The methanol and acetone extracts of *L. leonurus* inhibited all *K. pneumoniae* strains except strain [no. 5] (Table 7).

The *M. major* extracts displayed antibacterial activity against all the bacterial strains of *S. aureus* [no.'s 1 – 7], MRSA [no.'s 1 – 8] and *P. aeruginosa* [no.'s 1 – 5] (Table 7). The methanol extracts of *M. major* inhibited all thirteen *A. baumannii* strains [no.'s 1 – 13]. The acetone extract of *M. major* inhibited all except two [no.'s 1 & 4] strains and the aqueous extract inhibited all except four [no.'s 6, 7, 9 & 12] strains of *A. baumannii* strains (Table 7). The extracts of *M. major* inhibited all except one [no. 1] (aqueous extract) of the eight *K. pneumoniae* strains.

The extracts of *M. major* inhibited the highest number of bacterial strains in comparison to the other plant extracts. The methanol extract of *M. major* inhibited all 41 bacterial strains, the acetone extract inhibited 39 bacterial strains and the aqueous extract inhibited 36 bacterial strains.

The methanol extract of *Z. aethiopica* inhibited all seven [no.'s 1 – 7] *S. aureus* strains and six [no.'s 1, 4 – 8] of the eight MRSA bacterial strains. The aqueous extract of *Z. aethiopica* inhibited four [no.'s 2, 3, 6 & 7] of the seven *S. aureus* strains and all except one [no. 5] of the eight MRSA strains. The acetone extract of *Z. aethiopica* inhibited all except one [no. 1] of the seven *S. aureus* strains. Five [no.'s 1 & 5 – 8] of the eight MRSA strains were inhibited by the acetone *Z. aethiopica* extract (Table 7).

The methanol and acetone extracts of *Z. aethiopica* inhibited all four of the tested *P. aeruginosa* strains [no.'s 1 – 4]. The aqueous extract inhibited three [no.'s 1, 3 & 4] of the four tested *P. aeruginosa* strains. Only four [no.'s 1, 8, 11 & 12] of the tested *A. baumannii* strains were inhibited by the methanol extract of *Z. aethiopica* while the aqueous extract inhibited three strains [no.'s 1, 11 & 12] and the acetone extract inhibited four strains [no.'s 1, 6, 11 & 12]. The eight strains [no.'s 1 – 8] of *K. pneumoniae* were all inhibited by the methanol extracts of *Z. aethiopica* and all except one [no. 6] of the eight strains were inhibited by the aqueous and acetone extracts (Table 7).

The three extracts of *M. major* inhibited all 15 Gram-positive bacterial strains while the extracts of *B. frutescens* only inhibited a maximum of three bacterial strains for the Gram-positive bacteria. The methanol extract of *M. major* inhibited all 26 Gram-negative bacterial strains. The aqueous extract of *B. frutescens* inhibited 11 of the 26 Gram-negative bacterial strains, the least amount of Gram-negative bacteria inhibited in comparison to the other plant extracts.

TABLE 7 Screening results of medicinal plants for antibacterial activity

Bacteria tested: [Strains] Antibacterial activity of plants extracts tested against bacterial strains using the microtitre plate assay :													
		<i>Bulbine frutescens</i>			<i>Leonotis leonurus</i>			<i>Melianthus major</i>			<i>Zantedeschia aethiopica</i>		
		MeOH	Aqu.	Acet.	MeOH	Aqu.	Acet.	MeOH	Aqu.	Acet.	MeOH	Aqu.	Acet.
Gram + ve: <i>S. aureus</i>	[1]	+	+	-	+	+	+	+	+	+	+	-	-
	[2]	-	-	-	+	+	+	+	+	+	+	+	+
	[3]	+	-	+	-	+	+	+	+	+	+	+	+
	[4]	+	+	+	+	+	+	+	+	+	+	-	+
	[5]	NT	NT	NT	+	-	+	+	+	+	+	-	+
	[6]	-	-	-	+	+	+	+	+	+	+	+	+
	[7]	-	+	-	+	+	+	+	+	+	+	+	+
MRSA	[1]	-	-	-	+	+	+	+	+	+	+	+	+
	[2]	-	-	-	+	+	+	+	+	+	-	+	-
	[3]	-	-	-	+	+	+	+	+	+	-	+	-
	[4]	-	-	-	-	+	+	+	+	+	+	+	-
	[5]	-	-	-	+	+	+	+	+	+	+	-	+
	[6]	-	-	-	+	+	+	+	+	+	+	+	+
	[7]	-	-	-	+	+	-	+	+	+	+	+	+
	[8]	-	-	-	+	+	+	+	+	+	+	+	+
Gram – ve: <i>P. aeruginosa</i>	[1]	-	-	-	+	-	+	+	+	+	+	+	+
	[2]	-	-	-	+	+	+	+	+	+	+	-	+
	[3]	-	+	+	+	+	+	+	+	+	+	+	+
	[4]	+	-	+	+	-	+	+	+	+	+	+	+
	[5]	+	+	-	+	+	+	+	+	+	NT	NT	NT

TABLE 7 *Continues:*

<i>A. baumannii</i>	[1]	+	+	+	-	-	-	+	+	+	+	+	+
	[2]	-	-	-	+	+	+	+	+	+	-	-	-
	[3]	-	-	-	-	-	-	+	+	+	-	-	-
	[4]	+	+	+	+	-	+	+	+	+	-	-	-
	[5]	-	-	-	+	-	-	+	+	+	-	-	-
	[6]	+	-	+	+	-	-	+	-	+	-	-	+
	[7]	-	-	-	+	-	+	+	-	+	-	-	-
	[8]	+	-	+	+	-	+	+	+	+	+	-	-
	[9]	-	-	-	+	-	+	+	-	+	-	-	-
	[10]	+	-	-	+	-	+	+	+	-	-	-	-
	[11]	+	-	-	+	+	+	+	+	+	+	+	+
	[12]	-	-	-	+	+	+	+	-	+	+	+	+
	[13]	-	-	-	+	+	+	+	+	-	NT	NT	NT
<i>K. pneumoniae</i>	[1]	+	+	+	+	+	+	+	-	+	+	+	+
	[2]	+	+	-	+	+	+	+	+	+	+	+	+
	[3]	+	+	+	+	+	+	+	+	+	+	+	+
	[4]	+	+	+	+	+	+	+	+	+	+	+	+
	[5]	+	-	+	-	+	-	+	+	+	+	+	+
	[6]	+	+	+	+	+	+	+	+	+	+	-	-
	[7]	+	+	+	+	+	+	+	+	+	+	+	+
	[8]	+	+	+	+	+	+	+	+	+	+	+	+

(+) : Inhibition; (-): No inhibition; (NT): Not Tested

5.1.5 Standard antimicrobial sensitivity patterns of Gram-positive and Gram-negative bacteria

All the bacterial strains used in this study were clinical isolates collected from the NHLS (Main Branch) Port Elizabeth. Antibiotic sensitivity patterns were received with all of the isolates. The MIC's of the plant extracts were determined using a selection of these bacterial strains. Table 8a & 8b display the antibiotic sensitivity patterns of the selected Gram-positive and Gram-negative bacterial strains, respectively.

The following ten antibiotics were used for sensitivity testing of the Gram-positive bacteria namely:

penicillin (P10), co-amoxicillin (AMC30), cloxacillin (OX1), ofloxacin (OFX50), erythromycin (E15), clindamycin (DA2), vancomycin (VA30), fucidin (FD10), cotrimoxazole (FXT25), and tetracycline (TE30) (Table 8a).

All the *S. aureus* strains were resistant to penicillin and sensitive to the rest of the tested antibiotics except for strain [no. 4] that was resistant to erythromycin (Table 8a).

All the MRSA strains were resistant to penicillin, co-amoxicillin, cloxacillin, and erythromycin but sensitive to the other antibiotics tested except strain [no. 3] that was resistant to cotrimoxazole (Table 8a).

All the strains of *S. pyogenes* were sensitive to all the antibiotics (penicillin, erythromycin, cotrimoxazole and tetracycline) tested. All *E. faecalis* strains similar to *S. pyogenes* were sensitive to penicillin and erythromycin however; all the *E. faecalis* strains were resistant to cotrimoxazole and tetracycline (Table 8a).

Table 8b displayed the antibiotic sensitivity patterns of the Gram-negative bacterial strains with the following ten antibiotics:

ampicillin (AML10), co-amoxicillin (AMC30), cefazolin (KZ30), cefuroxime (CXM30), ceftriaxone (CRO30), ceftazidime (CAZ30), gentamycin (GN 10), amikacin (AK30), ofloxacin (OFX5) & cotrimoxazole (FXT25).

All three *P. aeruginosa* strains [no.'s 1 – 3] were resistant to gentamycin, while only two strains [no.'s 2 & 3] were resistant to ofloxacin. All three *P. aeruginosa* strains were sensitive to amikacin and two of the tested strains [no.'s 1 & 2] to ceftazidime.

The five *A. baumannii* strains were all resistant to cefazolin and cefuroxime. *A. baumannii* strains [no.'s 3 & 4] were completely resistant to all the tested antibiotics, followed by strain [no. 2] that was resistant to all the antibiotics except one antibiotic, gentamycin. *A. baumannii* [no. 1] was resistant to four antibiotics (co-amoxicillin, ceftriaxone, amikacin & cotrimoxazole) and sensitive to the rest of the antibiotics. ACBA [no. 5] was only resistant to two antibiotics (cefazolin & cefuroxime) and sensitive to the rest of the tested antibiotics (Table 8b).

All the *K. pneumoniae* strains [no.'s 1 – 5] were resistant to ampicillin and cotrimoxazole with varying sensitivity and resistance to the other antibiotics (Table 8b). In addition to ampicillin and cotrimoxazole resistance, *K. pneumoniae* strain [no. 1] were resistant to cefazolin and strain [no. 2] were resistant to four (co-amoxicillin, ceftriaxone, gentamycin & amikacin) antibiotics. *K. pneumoniae* strain [no. 3] was only resistant to ampicillin and cotrimoxazole while strain [no. 4] was resistant to an additional two antibiotics (co-amoxicillin, cefazolin). *K. pneumoniae* strain [no. 5] was only sensitive to one antibiotic (co-amoxicillin) and resistant to the rest of the nine antibiotics (Table 8b).

All the strains of *P. mirabilis* were resistant to ampicillin and cotrimoxazole except for *P. mirabilis* strain [no. 3] that was only resistant to ampicillin (Table 8b). *P. mirabilis* strain [no. 1] was resistant to an additional antibiotic (co-amoxicillin) and strain [no. 2] to additional two antibiotics (gentamycin & amikacin). *P. mirabilis* strain [no. 4] was only sensitive to two (co-amoxicillin & ofloxacin) antibiotics and resistant to the rest (Table 8b). *P. mirabilis* strain [no. 5] was resistant to five of the seven antibiotics tested for this strain and sensitive to the remaining two antibiotics (co-amoxicillin & cefazolin).

TABLE 8a Standard antimicrobial sensitivity patterns of Gram-positive bacterial strains selected for MIC determination

Gram + ve bacteria	[Strains]	Sensitivity results of standard antibiotics.									
		P10	AMC30	OX1	OFX5	E15	DA2	VA30	FD10	FXT25	TE30
<i>S. aureus</i>	[1]	R	S	S	S	S	S	S	S	S	-
	[2]	R	S	S	S	S	S	S	S	S	-
	[3]	R	S	S	S	S	S	S	S	S	-
	[4]	R	-	S	-	R	S	S	S	S	-
MRSA	[1]	R	R	R	S	R	S	S	S	S	-
	[2]	R	R	R	S	R	S	S	S	S	-
	[3]	R	R	R	S	R	S	S	S	R	-
	[4]	R	R	R	S	R	S	S	S	S	-
<i>S. pyogenes</i>	[1]	S	-	-	-	S	-	-	-	S	S
	[2]	S	-	-	-	S	-	-	-	S	S
	[3]	S	-	-	-	S	-	-	-	S	S
	[4]	S	-	-	-	S	-	-	-	S	S
	[5]	S	-	-	-	S	-	-	-	S	S
<i>E. faecalis</i>	[1]	S	-	-	-	S	-	-	-	R	R
	[2]	S	-	-	-	S	-	-	-	R	R
	[3]	S	-	-	-	S	-	-	-	R	R
	[4]	S	-	-	-	S	-	-	-	R	R
	[5]	S	-	-	-	S	-	-	-	R	R

R: Resistant
S: Sensitive
(-): Not tested

P10: Penicillin;
AMC30: Co-amoxicillin;
E15: Erythromycin;
FD10: Fucidin;

OX1: Cloxacillin;
OFX5: Ofloxacin;
FXT25: Cotrimoxazole;
TE30: Tetracycline;

DA2: Clindamycin
VA30: Vancomycin

TABLE 8b Standard antimicrobial sensitivity patterns of Gram-negative bacterial strains selected for MIC determination

Gram - ve bacteria	[Strains]	Sensitivity results of standard antibiotics.									
		AML10	AMC30	KZ30	CXM30	CRO30	CAZ30	GN10	AK30	OFX5	FXT25
<i>P. aeruginosa</i>	[1]	-	-	-	-	-	S	R	S	S	-
	[2]	-	-	-	-	-	S	R	S	R	-
	[3]	-	-	-	-	-	-	R	S	R	-
<i>A. baumannii</i>	[1]	R	S	R	R	S	-	R	S	-	S
	[2]	R	R	R	R	R	R	S	R	-	R
	[3]	R	R	R	R	R	R	R	R	-	R
	[4]	R	R	R	R	R	R	R	R	-	R
	[5]	S	S	R	R	S	S	S	S	-	S
<i>K. pneumoniae</i>	[1]	R	S	R	S	S	S	S	S	S	R
	[2]	R	R	S	S	R	-	R	R	S	R
	[3]	R	S	S	-	-	-	S	S	S	R
	[4]	R	R	R	S	S	S	S	S	S	R
	[5]	R	S	R	R	R	R	R	R	R	R
<i>P. mirabilis</i>	[1]	R	R	S	S	S	S	S	S	S	R
	[2]	R	S	S	S	S	S	R	R	S	R
	[3]	R	S	S	S	S	S	S	S	S	S
	[4]	R	S	R	R	R	R	R	R	S	R
	[5]	R	S	S	-	-	-	R	R	R	R

R: Resistant
S: Sensitive
(-): Not tested

AML10: Ampicillin;
AMC30: Co-amoxicillin;
CAZ30: Ceftazidime;
FXT25: Cotrimoxizole;

CXM30: Cefuroxime;
KZ30: Cefazolin;
AK30: Amikacin;

GN10: Gentamycin
CRO30: Ceftriaxone;
OFX5: Ofloxacin;

5.1.6 Plant extract dilutions that displayed antibacterial activity

The undiluted plant extracts of *Bulbine frutescens*, *Leonotis leonurus*, *Melianthus major* & *Zantedeschia aethiopica* were serially diluted in the microtitre plate assay to obtain the MIC's of the different plant extracts. Table 9a displays the dilutions where the MIC values were obtained for the specific plant extracts.

Twenty-one bacterial strains represented by two Gram-positive bacteria (*Staphylococcus aureus* & methicillin-resistant *Staphylococcus aureus*) and three Gram-negative bacteria (*Pseudomonas aeruginosa*, *Acinetobacter baumannii* & *Klebsiella pneumoniae*) were used for assessing the antibacterial activity of plant extracts in the microtitre plate assay.

The undiluted extract concentrations were different for each medicinal plant extract following resuspension of the extract residues with solvent. The extracts of *M. major* displayed the highest undiluted concentrations in comparison to the extracts of the other plants (Table 5). Therefore the dilutions in Table 9a represent different concentrations of the plant extracts where the MIC values (Table 9b) were obtained.

Table 9a indicates to what extent the plant extracts were diluted to display an inhibition of the bacteria in the microtitre plate assay. The dilutions in Table 9a correlate with the MIC values (mg/ml) in Table 9b. The specific plant extract dilutions in Table 9a serve as a point of reference for the visible observation of MIC's of plant extracts.

5.1.7 Minimal inhibitory concentrations of plant extracts

The microtitre plate assay was performed thrice and in triplicate in the microtitre plates, with all the medicinal plant (*Bulbine frutescens*, *Leonotis leonurus*, *Melianthus major* & *Zantedeschia aethiopica*) extracts for determination of the minimal inhibitory concentrations (MIC's).

The MIC's for medicinal plants were expressed as values (mg/ml) depending on the undiluted plant extract concentration and the lowest dilution that displayed an inhibition of the bacteria. Table 9b displays the plant extract concentration required to display an inhibitory effect for the tested bacterial strains.

P. aeruginosa and MRSA were not inhibited by any of the three *B. frutescens* extracts. *B. frutescens* only displayed MIC values at different concentrations for *S. aureus* and two strains [no.'s 1 & 4] of *A. baumannii* and *K. pneumoniae*, respectively (Table 9b).

The methanol extract of *B. frutescens* had an MIC of 0.26 mg/ml for all four *S. aureus* strains. The lowest and highest MIC values of *B. frutescens* against the Gram-positive bacteria were 0.13 mg/ml against *S. aureus* [no. 3] and 0.43 mg/ml against *S. aureus* [no.'s 1 & 2], respectively. The lowest MIC value of *B. frutescens* for the Gram-negative bacterial strains was the aqueous extract (0.05 mg/ml) against *K. pneumoniae* strain [no. 1] and the highest MIC was from the methanol and acetone extracts (0.51 mg/ml) against *K. pneumoniae* strain [no. 4] (Table 9b).

The extracts *L. leonurus* displayed antibacterial activity against all the bacterial strains of *S. aureus* and MRSA except MRSA strain [no. 4] (methanol extract). The lowest MIC value of *L. leonurus* against the Gram-positive bacterial strains was 0.07 mg/ml in the methanol extract against *S. aureus* strains [no.'s 2 & 3]. The highest MIC value of *L. leonurus* was 1.19 mg/ml in the aqueous extract against MRSA strain [no. 2] and *P. aeruginosa* strains [no.'s 3 & 4]. The lowest MIC value (0.03 mg/ml) for *L. leonurus* with the Gram-negative bacterial strains was obtained in the acetone and methanol extracts against *K. pneumoniae* strains [no.'s 1 & 2].

The extracts of *L. leonurus* inhibited four [no.'s 1 – 4] of the five *K. pneumoniae* strains. All the strains of *P. aeruginosa* were inhibited by the extracts of *L. leonurus* except strain [no. 1] with the aqueous extract and strain [no. 2] with the methanol and acetone extracts. Only *A. baumannii* strain [no. 2] was inhibited by all three extracts of *L. leonurus* and strain [no. 4] was inhibited by the methanol & acetone extracts (Table 9b).

M. major was the only plant that displayed antibacterial activity in all three extracts against all the Gram-positive and Gram-negative bacterial strains (Table 9b).

The lowest MIC value of *M. major* for Gram-positive bacterial strains was 0.03 mg/ml in the aqueous extract against *S. aureus* strains [no.'s 1, 2 & 4] and MRSA strains [1 – 4]. The MIC of 0.12 mg/ml in the methanol extract of *M. major* was the highest MIC against the Gram-positive bacterial strains, *S. aureus* strains [no.'s 3 & 4].

The lowest MIC value (0.03 mg/ml) of *M. major* against *P. aeruginosa* strains was in the acetone extract against strain [no. 2] and the highest MIC value (1.89 mg/ml) was in the methanol extract against strain [no. 3]. The lowest MIC value of the methanol extract of *M. major* was 0.03 mg/ml against *A. baumannii* strain [no. 1]. The highest MIC (1.66 mg/ml) of *M. major* against *A. baumannii* strains was in the aqueous extract against strain [no. 3]. The strains of *K. pneumoniae* displayed the highest (1.66 mg/ml) and lowest (0.05 mg/ml) MIC values of *M. major* against strain [no. 1] and strain [no. 4], respectively.

The methanol extracts of *Z. aethiopica* inhibited all the *S. aureus* strains [no.'s 1 – 4] and none of the MRSA strains. The aqueous extracts of *Z. aethiopica* only inhibited *S. aureus* [no. 3] and MRSA [no. 1] with MIC's of 0.01 mg/ml and 0.62 mg/ml, respectively. In comparison to the other plants, the aqueous extract of *Z. aethiopica* had the lowest MIC of 0.01 mg /ml.

Only one [no. 2] of the three *P. aeruginosa* strains was inhibited by the methanol (1.09 mg/ml) and aqueous (0.62 mg/ml) extracts. The acetone extracts of *Z. aethiopica* inhibited two *P. aeruginosa* strains [no.'s 2 & 3] with MIC's of 1.00 mg/ml and 0.25 mg/ml, respectively. Only one [strain no. 1] of the five *A. baumannii* strains was inhibited by the extracts of *Z. aethiopica* (Table 9a & 9b). The lowest and highest MIC's of *Z. aethiopica* against Gram-negative bacterial strains were 0.03

mg/ml (acetone extract) against *K. pneumoniae* strains [no.'s 1] and 1.09 mg/ml (methanol extract) against different strains, respectively (Table 9b).

Table 10, Fig 10 & 11 displays the highest and lowest MIC values for each plant extract against all the Gram-positive and Gram-negative bacterial strains. The undiluted plant extract concentrations were also included as a representation of the starting point of each extract and for comparison of the different MIC's (Table 10).

The lowest MIC's of *B. frutescens* for the Gram-positive bacterial strains were 0.13 mg/ml in the acetone extract and 0.05 mg/ml in the aqueous extract for Gram-negative bacterial strains. The highest MIC of *B. frutescens* for the Gram-positive bacterial strains was 0.43 mg/ml (aqueous extract), while the undiluted extract concentration of 0.51 mg/ml (acetone and methanol extracts) was the maximum MIC for the Gram-negative bacterial strains.

The lowest MIC's of *L. leonurus* for Gram-positive and Gram-negative bacterial strains were 0.07 mg/ml (methanol extract) and 0.02 mg/ml (aqueous and acetone extracts), respectively. The undiluted aqueous extract of 1.19 mg/ml was the highest MIC for both Gram-positive and Gram-negative bacterial strains.

The MIC's of 0.03 mg/ml (aqueous extract) and 0.03 mg/ml (methanol extract) were the lowest MIC values of *M. major* against the Gram-positive and Gram-negative bacterial strains, respectively (Fig. 10). The highest MIC values of *M. major* against the Gram-positive and Gram-negative bacterial strains were 0.21 mg/ml (aqueous extract) and 2.17 mg/ml (acetone extract), respectively (Table 10 & Fig. 11).

The aqueous extract of *Z. aethiopica* displayed the lowest MIC's of 0.01 mg/ml (aqueous extract) against Gram-positive bacterial strains and 0.03 mg/ml (acetone extract) against Gram-negative bacterial strains (Fig. 10). The highest MIC's of *Z. aethiopica* against Gram-positive bacterial strains were 1.00 mg/ml in the acetone extract and 1.09 mg/ml (methanol extract) against the Gram-negative bacterial strains (Fig. 11).

TABLE 9a Representation of the extract dilutions where the MIC's of medicinal plants was obtained for the bacterial strains

Bacteria tested:	[Strains]	Extract dilutions where MIC's were obtained for medicinal plants:											
		<u><i>Bulbine frutescens</i></u>			<u><i>Leonotis leonurus</i></u>			<u><i>Melianthus major</i></u>			<u><i>Zantedeschia aethiopica</i></u>		
		MeOH	Aqu.	Acet.	MeOH	Aqu.	Acet.	MeOH	Aqu.	Acet.	MeOH	Aqu.	Acet.
Gram + ve:													
<i>S. aureus</i>	[1]	1:2	UE	-	UE	1:2	1:2	1:64	1:64	1:64	1:2	-	-
	[2]	1:2	UE	-	1:16	1:2	1:16	1:64	1:64	1:64	1:2	-	1:2
	[3]	1:2	-	1:4	1:4	1:2	1:8	1:16	1:8	1:64	1:8	1:64	1:64
	[4]	1:2	1:2	-	1:2	1:4	1:4	1:16	1:64	1:64	1:8	-	1:4
MRSA	[1]	-	-	-	1:16	1:16	1:4	1:64	1:64	1:64	-	UE	UE
	[2]	-	-	-	1:2	UE	1:2	1:64	1:64	1:64	-	-	-
	[3]	-	-	-	1:8	1:16	1:2	1:64	1:64	1:64	-	-	-
	[4]	-	-	-	-	1:4	1:8	1:64	1:64	1:64	-	-	-
Gram – ve:													
<i>P. aeruginosa</i>	[1]	-	-	-	UE	-	UE	1:16	1:4	1:16	-	-	-
	[2]	-	-	-	-	UE	-	1:8	1:8	1:64	UE	UE	UE
	[3]	-	-	-	1:4	UE	1:2	UE	UE	1:4	-	-	1:4
<i>A. baumannii</i>	[1]	1:2	UE	1:8	-	-	-	1:64	1:4	1:64	UE	UE	UE
	[2]	-	-	-	UE	1:2	1:2	1:4	1:2	1:4	-	-	-
	[3]	-	-	-	-	-	-	1:2	UE	1:4	-	-	-
	[4]	1:2	UE	UE	1:2	-	1:16	1:4	1:2	1:16	-	-	-
	[5]	-	-	-	-	-	-	1:32	1:2	1:16	-	-	-
<i>K. pneumoniae</i>	[1]	1:8	1:8	1:8	1:64	1:16	1:64	1:8	UE	1:16	1:4	1:2	1:32
	[2]	-	-	-	1:64	1:64	1:16	1:8	1:4	1:16	-	-	-
	[3]	-	-	-	1:2	1:2	1:4	1:16	1:16	1:32	UE	1:16	1:16
	[4]	UE	UE	UE	1:4	1:2	1:4	1:32	1:32	1:16	UE	1:2	1:16
	[5]	-	-	-	-	-	-	1:4	1:2	1:4	1:2	-	-

(UE): Undiluted Extract; (-): No activity; MeOH: methanol; Aqu.: aqueous; Acet.: acetone; MRSA: methicillin-resistant *S. aureus*

TABLE 9b Minimal inhibitory concentrations of medicinal plant extracts for antibacterial testing

Bacteria tested:	[Strains]	Minimal inhibitory concentration of medicinal plants (mg/ml)											
		<u><i>Bulbine frutescens</i></u>			<u><i>Leonotis leonurus</i></u>			<u><i>Melianthus major</i></u>			<u><i>Zantedeschia aethiopica</i></u>		
		MeOH	Aqu.	Acet.	MeOH	Aqu.	Acet.	MeOH	Aqu.	Acet.	MeOH	Aqu.	Acet.
Gram + ve:													
<i>S. aureus</i>	[1]	0.26	0.43	-	1.05	0.60	0.56	0.03	0.03	0.03	0.55	-	-
	[2]	0.26	0.43	-	0.07	0.60	0.07	0.03	0.03	0.03	0.55	-	0.50
	[3]	0.26	-	0.19	0.26	0.60	0.14	0.12	0.21	0.03	0.14	0.01	0.02
	[4]	0.26	0.22	-	0.53	0.30	0.28	0.12	0.03	0.03	0.14	-	0.25
MRSA	[1]	-	-	-	0.07	0.07	0.28	0.03	0.03	0.03	-	0.62	1.00
	[2]	-	-	-	0.53	1.19	0.56	0.03	0.03	0.03	-	-	-
	[3]	-	-	-	0.13	0.07	0.56	0.03	0.03	0.03	-	-	-
	[4]	-	-	-	-	0.30	0.14	0.03	0.03	0.03	-	-	-
Gram – ve:													
<i>P. aeruginosa</i>	[1]	-	-	-	1.05	-	1.11	0.18	0.42	0.14	-	-	-
	[2]	-	-	-	-	1.19	-	0.24	0.21	0.03	1.09	0.62	1.00
	[3]	-	-	-	0.26	1.19	0.56	1.89	1.66	0.54	-	-	0.25
<i>A. baumannii</i>	[1]	0.26	0.43	0.06	-	-	-	0.03	0.42	0.03	1.09	0.62	1.00
	[2]	-	-	-	1.05	0.60	0.56	0.47	0.83	0.54	-	-	-
	[3]	-	-	-	-	-	-	1.95	1.66	0.54	-	-	-
	[4]	0.26	0.43	0.51	0.53	-	0.07	0.47	0.83	0.14	-	-	-
	[5]	-	-	-	-	-	-	0.06	0.83	0.14	-	-	-
<i>K. pneumoniae</i>	[1]	0.06	0.05	0.06	0.02	0.07	0.02	0.24	1.66	0.14	0.27	0.31	0.03
	[2]	-	-	-	0.02	0.02	0.07	0.24	0.42	0.14	-	-	-
	[3]	-	-	-	0.53	0.60	0.28	0.12	0.10	0.07	1.09	0.04	0.06
	[4]	0.51	0.43	0.51	0.26	0.60	0.28	0.06	0.05	0.14	1.09	0.31	0.06
	[5]	-	-	-	-	-	-	0.47	1.83	0.54	0.55	-	-

(-) : No activity;

MeOH: methanol;

Aqu.: aqueous;

Acet.: acetone;

MRSA: methicillin-resistant *S. aureus*

The lowest MIC's of *B. frutescens* and *L. leonurus* were higher for Gram-positive bacterial strains than for the Gram-negative bacterial strains (Table 10). The lowest MIC's of *M. major* and *Z. aethiopica* were higher for the Gram-negative bacterial strains. The highest MIC's of all the plants were higher for the Gram-negative bacterial strains than for the Gram-positive bacterial strains.

The highest MIC's of the plant extracts against the Gram-negative bacterial strains were the same as the undiluted plant extract concentrations. Therefore some Gram-negative bacterial strains were only inhibited in the initial wells of the microtitre plates containing undiluted plant extracts.

TABLE 10 The lowest and highest MIC's for each medicinal plant extract in the microtitre plate assay

Medicinal Plants:	Extracts:	Undiluted Extract (mg/ml)	MIC's (mg/ml) in microtitre plate assay.			
			Gram +ve bacteria:		Gram –ve bacteria:	
			Low.	High.	Low.	High.
<i>B. frutescens</i>	Acet	0.51	0.13	0.13	0.06	0.51
	MeOH	0.51	0.26	0.20	0.06	0.51
	Aqu.	0.43	0.22	0.43	0.05	0.43
<i>L. leonurus</i>	Acet	1.11	0.07	0.56	0.02	1.11
	MeOH	1.05	0.07	1.05	0.02	1.05
	Aqu	1.19	0.07	1.19	0.07	1.19
<i>M. major</i>	Acet	2.17	0.03	0.03	0.03	2.17
	MeOH	1.89	0.03	0.12	0.03	1.89
	Aqu	1.66	0.03	0.21	0.05	1.66
<i>Z. aethiopica</i>	Acet	1.00	0.02	1.00	0.03	1.00
	MeOH	1.09	0.14	0.55	0.27	1.09
	Aqu	0.62	0.01	0.62	0.04	0.62

Low.: Lowest; High.: Highest; Acet: acetone; Aqu: Aqueous; MeOH: Methanol

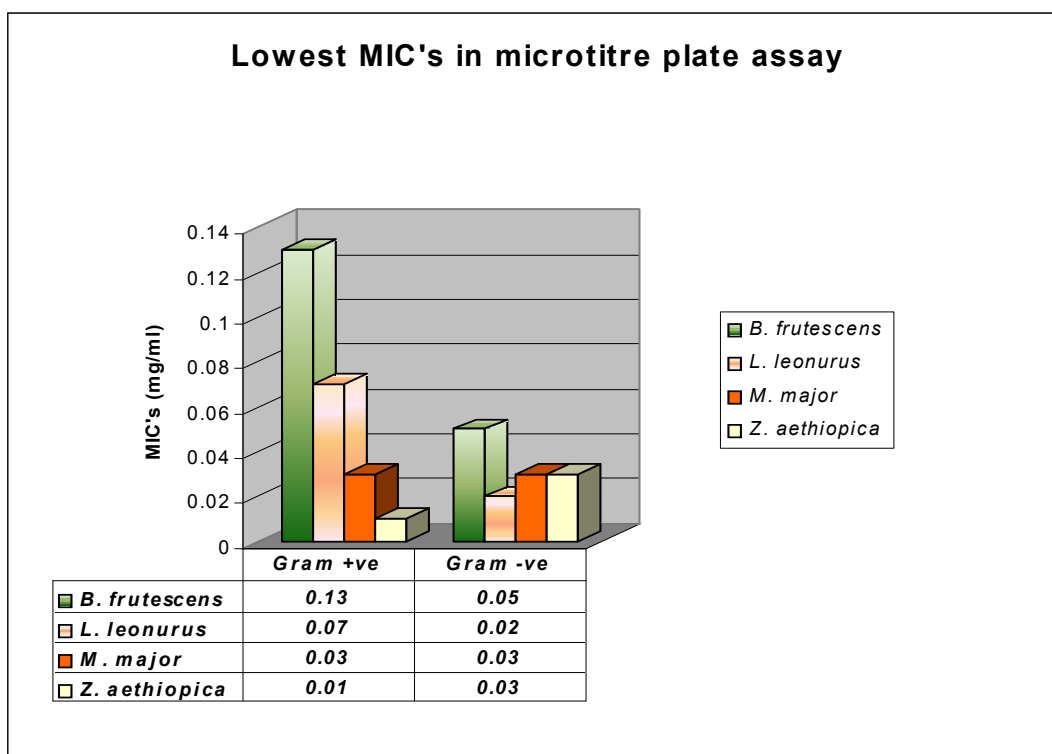


FIGURE 10 The lowest MIC values of the different medicinal plants in the microtitre plate assay

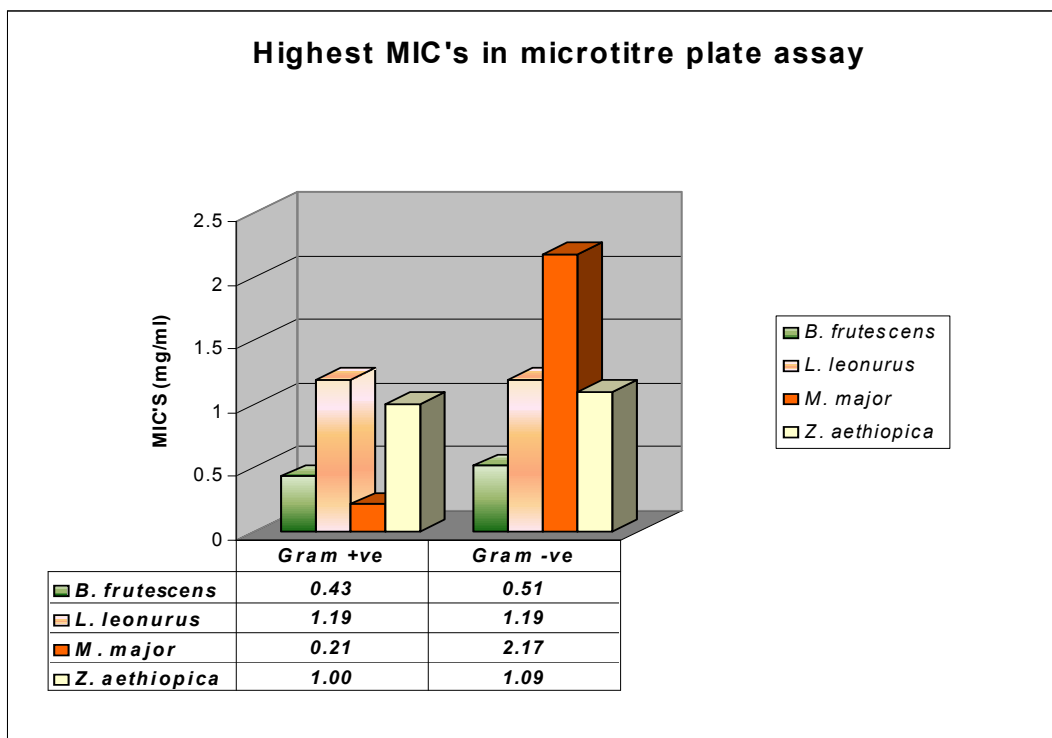


FIGURE 11 The highest MIC values of the different medicinal plants in the microtitre plate assay

5.1.8 The number of bacterial strains inhibited by the plant extracts

Twenty-one Gram-positive and Gram-negative bacterial strains from a total of 36 bacterial strains were used for determining the MIC's of the different plant extracts in the microtitre plate assay. The different plant extracts displayed different patterns of activity (Figure 12).

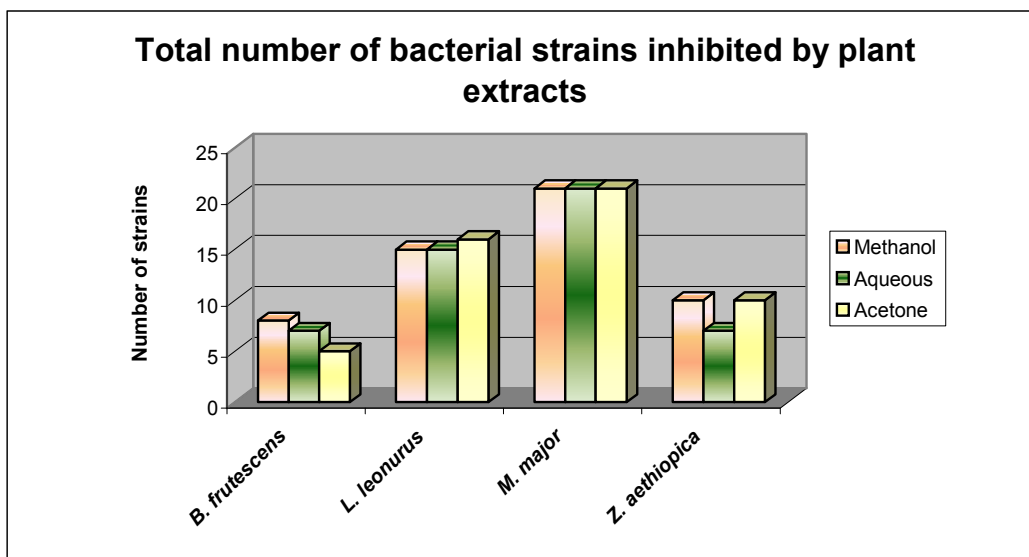


FIGURE 12 Number of bacterial strains inhibited by the plant extracts in the microtitre plate assay

All three extracts of *M. major* inhibited the total of 21 bacterial strains in the microtitre plate assay at varying concentrations (Table 9b) for each extract, respectively. The three respective extracts of *L. leonurus* inhibited the second highest numbers of bacterial strains from the total of 21 bacterial strains (Fig. 12). The plant extracts of *Z. aethiopica* followed by the extracts of *B. frutescens* inhibited the lowest number of bacteria tested. The extracts of *Z. aethiopica* inhibited a maximum of ten bacterial strains while *B. frutescens* only inhibited eight of the bacterial strains from the total of 21 bacterial strains tested (Figure 12).

The methanol extract of the three plants (*B. frutescens*, *M. major* & *Z. aethiopica*) inhibited the highest number of bacterial strains. The acetone extract of *L. leonurus* inhibited one more bacterial strain than the methanol extract of the plant. The methanol and acetone extracts of the plant displayed similar antibacterial activity with respect to the number of bacterial strains inhibited (Figure 12).

5.1.9 Antibacterial activity of traditional medicinal plant preparations

The traditional methods (infusions & decoctions) of preparing the medicinal plants for therapeutic purposes were tested for antibacterial activity using the microtitre plate assay. The antibacterial activity of traditional plant preparations (infusions & decoctions) was expressed as the extent of dilution displaying a bacterial inhibition. This displays to what extent the traditional plant preparations may be diluted and still display an inhibitory effect on the specific bacteria.

Ethnobotanical information revealed that traditionally *M. major* and *L. leonurus* are prepared as decoctions for treating infected skin conditions. Although no traditional preparations are recorded for the use of *B. frutescens* and *Z. aethiopica* in treating skin conditions, traditional preparations (infusion & decoction, respectively) were included. The infusion and decoction preparations of *B. frutescens* & *Z. aethiopica*, respectively were included to observe if these plants might have antibacterial activity if these plants were used in the form of a preparation instead of fresh.

The infusion and decoction preparations of *B. frutescens* and *Z. aethiopica* displayed very little antibacterial activity against the Gram-positive bacterial strains. *B. frutescens* did not inhibit any of the Gram-positive bacterial strains whereas the *Z. aethiopica* inhibited only one strain of *S. aureus* & MRSA, respectively (Table 11).

B. frutescens inhibited two *P. aeruginosa* strains [no.'s 1 & 2], two *A. baumannii* strains [no.'s 1 & 2] and four [no.'s 1 – 4] of the five *K. pneumoniae* strains. *Z. aethiopica* inhibited one *P. aeruginosa* strain [no. 2] and three *A. baumannii* strains [no.'s 1, 3 & 5] at the undiluted decoction concentrations. Two *K. pneumoniae* strains [no.'s 4 & 5] were inhibited by *Z. aethiopica* (Table 11).

The *L. leonurus* decoction inhibited all the bacterial strains except MRSA strain [no. 1] at different dilutions. The highest dilution of the *L. leonurus* decoction that displayed antibacterial activity for the Gram-negative bacterial strains was at the 1:32 dilution against *A. baumannii* strain [no. 3] (Table 10). The highest dilution that

displayed antibacterial activity against the Gram-positive bacterial strains was at the 1:16 dilution for MRSA strain [no. 2].

Diluted concentrations of the *M. major* decoction inhibited all MRSA strains [no.'s 1 – 4] at the 1:64 dilution of the decoction. *M. major* inhibited all *S. aureus* [no.'s 1 – 4], *P. aeruginosa* [no.'s 1 – 3] and *A. baumannii* [no.'s 1 – 5] strains at varying dilutions (Table 11). Four [no.'s 2 – 5] of the five *K. pneumoniae* strains were inhibited at different dilutions of the *M. major* decoction preparation. *M. major* inhibited *P. aeruginosa* strain [no. 2] and *A. baumannii* strain [no. 1] in the last dilution (1:64) of the plant decoction.

TABLE 11 Dilutions that displayed antibacterial activity for traditional plant preparations

Bacteria tested:		[Strains:] Dilutions that displayed antibacterial activity:			
		<i>B. frutescens</i> [Infusion]	<i>L. leonurus</i> [Decoction]	<i>M. major</i> [Decoction]	<i>Z. aethiopica</i> [Decoction]
Gram + ve:					
<i>S. aureus</i>	[1]	-	1:2	1:8	-
	[2]	-	1:4	1:64	-
	[3]	-	N	1:16	1:4
	[4]	-	1:4	1:8	-
MRSA	[1]	-	-	1:64	-
	[2]	-	1:16	1:64	-
	[3]	-	1:4	1:64	1:4
	[4]	-	1:2	1:64	-
Gram – ve:					
<i>P. aeruginosa</i>	[1]	UE	1:8	1:32	-
	[2]	1:2	1:4	1:64	UE
	[3]	-	1:8	UE	-
<i>A. baumannii</i>	[1]	1:2	1:4	1:64	UE
	[2]	UE	1:2	1:16	-
	[3]	-	1:32	1:8	UE
	[4]	-	1:4	1:16	-
	[5]	-	1:8	1:32	UE
<i>K. pneumoniae</i>	[1]	1:2	1:16	-	-
	[2]	UE	1:4	1:32	-
	[3]	1:16	1:16	1:8	-
	[4]	1:4	1:2	1:16	1:16
	[5]	-	1:2	1:32	UE

(UE): Undiluted Extract; (-): No Activity.

5.2 Agar dilution assay

5.2.1 Antibacterial activity screening and MIC determination of plant extracts

The agar dilution assay was used for screening and MIC determination of the plant extracts against the bacterial strains of *S. pyogenes*, *E. faecalis* and *P. mirabilis*.

The agar plate contained extract concentrations of 10.0 mg/ml, 5.0 mg/ml, 1.0 mg/ml and 0.5 mg/ml for antibacterial activity testing.

The different plant extracts did not inhibit any of the *S. pyogenes* & *E. faecalis* strains. The *M. major* was the only plant that displayed antibacterial activity. *Proteus mirabilis* was the only bacteria inhibited by the acetone and methanol extracts of *M. major* at concentrations of 5.0 and 10.0 mg/ml. No other inhibitions were observed with any extracts and plants (Table 12). The *M. major* extracts did not inhibit any of the bacteria at the lower concentrations of 1.0 & 0.5 mg/ml. The acetone and methanol extracts of *M. major* had MIC's of 5.0 mg/ml against the strains of *P. mirabilis* (Table 12).

Further tests were performed on the methanol extract of *M. major* at 10 mg/ml against *P. mirabilis*. The purpose was to compare the difference between fresh leaves and leaves stored in the refrigerator for a few days prior to extraction. The extracts of the stored leaves did not inhibit all the strains of *P. mirabilis* (Figure 13). Extracts obtained from fresh leaves inhibited all the *P. mirabilis* strains at 5 mg/ml (Figure 13) as well as higher extract concentrations (10 & 15 mg/ml).

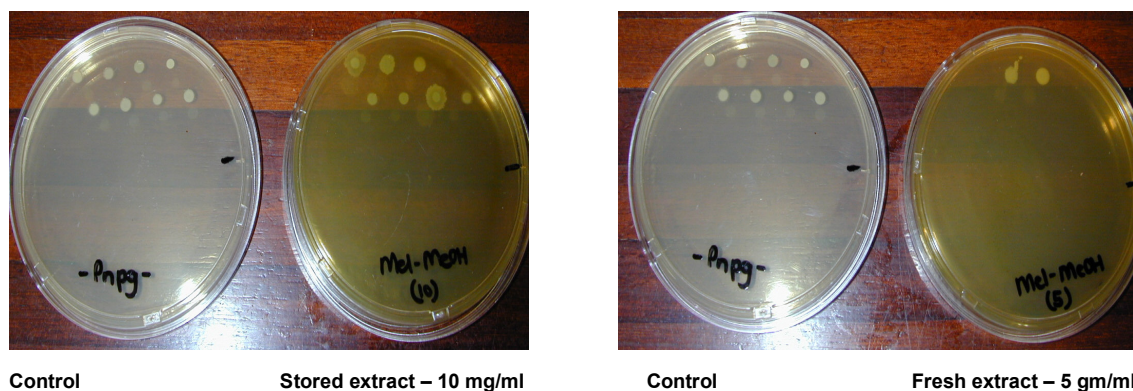


FIGURE 13 Antibacterial activity of fresh and stored extracts of *M. major*

TABLE 12 Antibacterial activity of plant extracts in the agar dilution assay

Medicinal plants:	Extracts:	Conc. mg/ml	Antibacterial activity of plant extracts		
			<i>S. pyogenes</i> [5 strains]	<i>E. feacalis</i> [5 strains]	<i>P. mirabilis</i> [5 strains]
<i>B. frutescens</i>	Methanol	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-
	Aqueous	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-
	Acetone	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-
<i>L. leonurus</i>	Methanol	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-
	Aqueous	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-
	Acetone	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-
<i>M. major</i>	Methanol	10.0	-	-	+
		5.0	-	-	+
		1.0	-	-	-
		0.5	-	-	-
	Aqueous	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-
	Acetone	10.0	-	-	+
		5.0	-	-	+
		1.0	-	-	-
		0.5	-	-	-
<i>Z. aethiopica</i>	Methanol	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-
	Aqueous	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-
	Acetone	10.0	-	-	-
		5.0	-	-	-
		1.0	-	-	-
		0.5	-	-	-

(-): No Inhibition;

(+): Inhibition

The acetone and methanol extracts of *M. major* only inhibited *P. mirabilis* strains at concentrations of 5.0 and 10.0 mg/ml (Table 12). Therefore increased concentrations of these *M. major* extracts at 15.0 and 20.0 mg/ml were tested.

The acetone and methanol *M. major* extracts did not inhibit the strains of *S. pyogenes* and *E. feacalis* at the concentrations of 15.0 and 20.0 mg/ml (Table 13). All the strains of *P. mirabilis* [no.'s 1 – 5] were inhibited at the increased concentrations of the acetone and methanol extracts of *M. major* (Table 13 & Fig. 14).

TABLE 13 Higher concentrations of the acetone and methanol extracts of *M. major* tested for antibacterial activity

<i>M. major</i> extracts (mg/ml)	Antibacterial activity of plant extracts:		
Acetone & Methanol	<i>P. mirabilis</i> [5 strains]	<i>S. pyogenes</i> [5 strains]	<i>E. feacalis</i> [5 strains]
0.5	-	-	-
1.0	-	-	-
5.0	+	-	-
10	+	-	-
15	+	-	-
20	+	-	-
(-): No inhibition (+): inhibition			

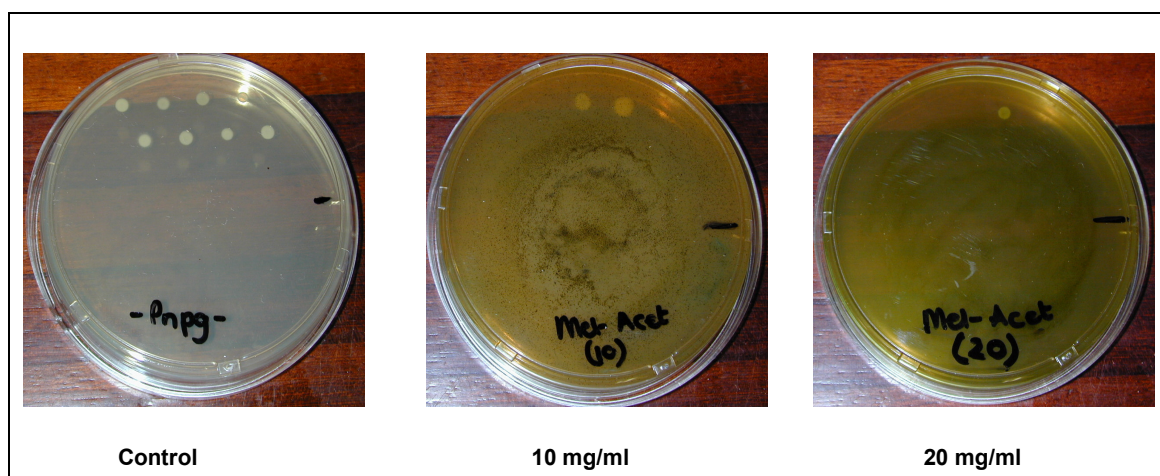


FIGURE 14 Antibacterial activity of acetone extracts of *M. major* against *P. mirabilis* at higher concentrations

5.2.2 Antibacterial activity of traditional plant preparations

Traditional preparations, *Leonotis leonurus* & *Melianthus major* were prepared as decoctions for antibacterial activity testing. Infusions of *B. frutescens* leave gel and decoctions of *Z. aethiopica* were also included for testing against the strains of the three bacteria.

The *B. frutescens* infusion and decoctions of the other three plants (*L. leonurus*, *M. major* & *Z. aethiopica*) did not inhibit any of the bacterial strains. Therefore no dilutions of the traditional preparations were tested.

TABLE 14 Antibacterial activity of traditional plant preparations on agar plates

Medicinal plants:	Traditional preparations:	Bacterial strains:		
		<i>P. mirabilis</i> [5 strains]	<i>S. pyogenes</i> [5 strains]	<i>E. feacalis</i> [5 strains]
<i>B. frutescens</i>	Infusion	-	-	-
<i>L. leonurus</i>	Decoction	-	-	-
<i>M. major</i>	Decoction	-	-	-
<i>Z. aethiopica</i>	Decoction	-	-	-

(-): No inhibition on plates; (+): Inhibition

5.3 Standard agar plate count technique

The relative growth percentages of selected bacteria were more than 100% in the test plant extract wells. This indicated that the growth of these bacteria was stimulated at the specific plant extract concentrations in the microtitre plate assay.

Selected specific bacterial strains that displayed a relative growth percentage of more than 100% were compared to one that showed a growth percentage of less than 100% with the specific plant extract concentrations.

Bacterial strains with growth percentages of more than 100% selected for testing included MRSA [no. 3] with the acetone extract of *Z. aethiopica* and *P. aeruginosa* [no. 1], *K. pneumoniae* [no. 3] & *A. baumannii* [no. 5] with the acetone extract of *M. major* (Table 15). *S. aureus* [no. 1] with the growth percentage less than 100% were included for comparative purposes (Table 15).

The bacteria with growth percentages of more than 100%, all displayed higher cfu/ml in the test extract wells in comparison to the controls (Table 15). *S. aureus* [no. 1] with the relative growth percentage of 91%, displayed lower cfu/ml in plant extract wells in comparison to the control (Fig. 15).

Results in Table 15 and Fig. 15 indicated that bacterial growth percentages of more than 100% stimulated growth slightly and growth percentages of less than 100% inhibited bacterial growth at the specific plant extract concentrations (Table 15).

The results confirmed that if the relative growth percentages of bacterial strains are more than 100% it appears to be stimulated at the specific plant extract concentration. Further investigation is needed into this phenomenon.

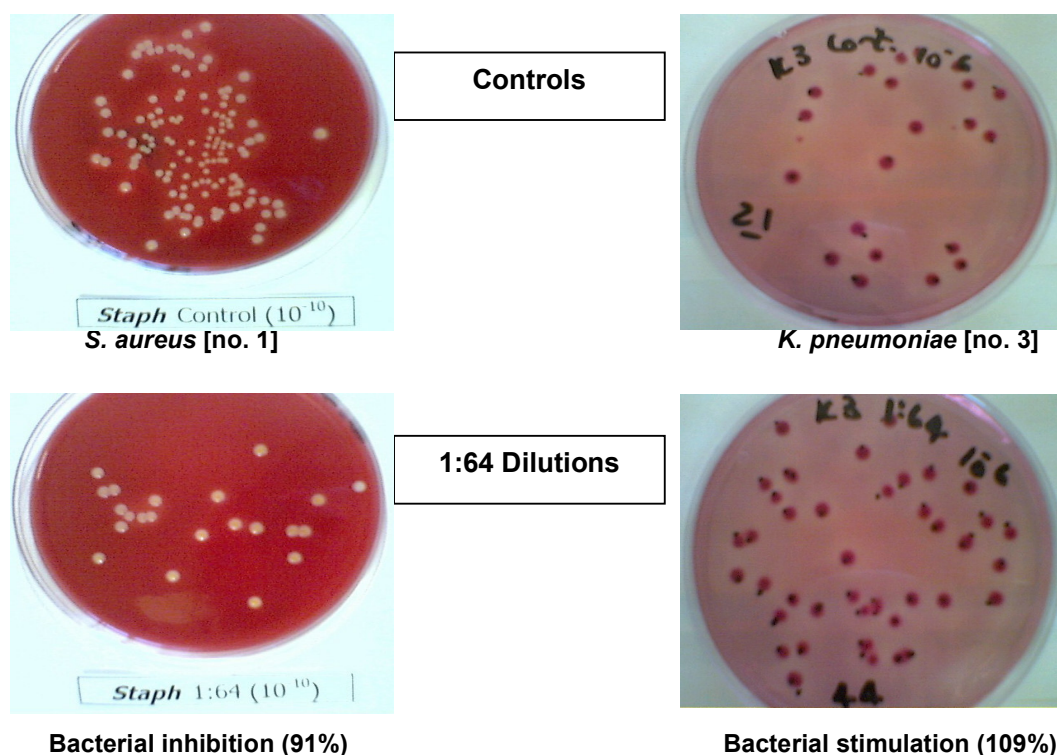


FIGURE 15 Representations of bacterial colony forming units/ml on agar plates

TABLE 15 Colony forming units (cfu/ml) of microtitre plate bacterial suspensions

Selected bacterial [strains]:	Plant extract suspensions:	Comparison of bacterial growth			
		Microtitre plate assay		Standard agar plate count	
		Growth %	Microtitre plate Activity	Cfu/ml from dilutions of microtitre plate suspensions	
		1:64	1:64	1:64	Bacteria control
<i>S. aureus</i> [no. 1]	<i>M. major</i> (Acetone)	91%	+	1.30×10^{11}	1.34×10^{11}
MRSA [no. 3]	<i>Z. aethiopica</i> (Acetone)	111%	-	8.70×10^{11}	0.57×10^{11}
<i>P. aeruginosa</i> [no. 1]	<i>M. major</i> (Acetone)	105%	-	4.40×10^7	2.10×10^7
<i>K. pneumoniae</i> [no. 3]	<i>M. major</i> (Acetone)	109%	-	1.86×10^9	1.18×10^9
<i>A. baumannii</i> [no. 5]	<i>M. major</i> (Acetone)	106%	-	1.00×10^7	0.70×10^7

(+): Inhibition; (-): No inhibition

CHAPTER 6

DISCUSSION AND CONCLUSION

The four medicinal plants selected for investigation in this study form part of the traditional medicinal plants used in the Eastern Cape as well as other parts of South Africa. These plants are indigenously used for treating skin conditions by topical applications onto the affected area.

The growing demand and popularity of medicinal plants in rural as well as urban communities (Matsiliza & Barker, 2001) has placed many medicinal plants under the threat of extinction. Therapeutic use of medicinal plants is an alternative health care option for those living in urban communities, but the only means of healing for some people in rural communities.

In rural communities the therapeutic use of medicinal plants are mainly governed by economic factors, ease of availability and the strong belief in the plant remedies. The use of medicinal plants for treating various ailments ranging from acute to chronic conditions has become a way of life for many indigenous people in rural communities. Based on regular usage, many indigenous people are familiar with the different uses, preparations and identification of medicinal plants. Traditional herbalists and herb-sellers are the main sources of distributing information and prescriptions on medicinal plant remedies in rural communities (Matsiliza & Barker, 2001).

The therapeutic use of medicinal plants in urban communities is mainly encouraged by the adoption of a more holistic approach in maintaining health. People in the urban communities are familiar with medicinal plants in the form of purified plant extracts, capsules and tonics that are sold as over-the-counter herbal formulations. Herbal shops and pharmacies provide these purified medicinal plant extracts and information regarding the uses to people in urban communities (Kong *et al.*, 2003).

The growing threat and spread of antibiotic resistance by a wide range of common pathogens has led to increased investigations into traditional medicinal plants as alternatives. Antibiotic resistance are not selective in that antibiotic resistance with the same consequences may affect people living in urban and rural communities around the world. Antibiotics that once readily cured a wide range of infections are becoming less useful mainly due to the misuse of antibiotics and the development of antibiotic resistance (Nostro *et al.*, 2000).

Although new leads for effective antimicrobials are researched in the plant kingdom, education on the proper use of antimicrobial agents is needed. The development of antibiotic resistance was not anticipated with the development and widespread use of synthetic antibiotics. Therefore it may be wise not to follow the same path with the development of purified medicinal plant extracts used as antibiotics. Herbal remedies prepared from the whole plant are generally safe with fewer side effects if it is used in the proper therapeutic dosages (Hanrahan, 2001). The proper use of standardized herbal extracts and antibiotics that are currently still effective may help in eliminating infections without the development of antimicrobial resistance.

The main aim of the study was to establish the scientific validity of the traditional uses of these plants for treating wound infections. The information regarding the presence or absence of antibacterial activity may enhance the use of efficacious indigenous plant remedies.

The antibacterial activity (Table 7, 9b & 12) of the plant extracts against the different clinical strains of burn wound pathogens supported the scientific validity of the plants being used traditionally for treating wounds infected with these bacteria.

Establishing the antibacterial activity of the plant extracts also contributed on a whole to the scientific investigation done on indigenous medicinal plants in South Africa. According to George and van Staden (2000) collaborative ventures with mutual understanding on the returns are needed for widespread screening of medicinal plants.

The selection and standardization of an appropriate plant extraction procedure is essential as it may influence the results of a scientific investigation on medicinal plants (Nostro *et al.*, 2000). George *et al.* (2001) suggested that the traditional

methods of processing medicinal plants be taken into account when standardizing procedures for scientific research. Ethnobotanical information revealed that all the plants selected in this study are traditionally used fresh for medicinal purposes (Van Wyk *et al.*, 1997; Rabe & van Staden, 1997; Grierson & Afolayan, 1999b).

The incomplete and problematic drying of the aqueous extract supernatants in the fume-cupboard prompted alternative drying in an incubator (37°C) to obtain a dried extract residue for analysis (George *et al.*, 2001). Although many studies report on using the respective solvents for redissolving the dried extract residues (Pillay *et al.*, 2001; Eloff, 1999), in this study it was found to be problematic, especially with the use of acetone.

A visual inspection method was used for assessing the antibacterial activity of plant extracts in studies using the respective solvents as a redissolving agent (Eloff, 1999). However using high concentrations of extracts redissolved in acetone interfered with the transparency of microtitre plates and the subsequent spectrophotometric analysis of the microtitre plates in this study.

Therefore dimethylsulphoxide (DMSO) was used for redissolving the solvent-free dried plant extract residues (Harsha *et al.*, 2003; Nostro *et al.*, 2000). The freshly resuspended acetone and methanol extracts in DMSO, posed problems with subsequent spectrophotometric analysis, due to chlorophyll interference of the dark coloured extracts. The colour interference was circumvented with the preparation of a 1.25% solution in distilled water of each stock DMSO plant extract.

The different chemical compositions of the plants may have contributed to the processing of medicinal plants into fine leave particles. Leaves of *Melianthus major* followed by *Leonotis leonurus* produced very fine leave particles in comparison to the of *Zantedeschia aethiopica* that did not produce as fine leave particles after crushing with a pestle and mortar.

The evergreen leaves of *Z. aethiopica* are naturally glossy which contributed to the difficulty experienced with crushing the leaves into fine particles prior to extraction. The glossy leaves of *Z. aethiopica*, failed to produce fine leave particles after trial runs using a blender for processing the leaves. Plant material of *Bulbine frutescens*

was presented for extraction as a semi-transparent leave gel obtained by removing most of the outer green layer. Difficulty was occasionally experienced in obtaining large amounts of the *B. frutescens* leave gel for extraction.

M. major (1.91mg/ml) followed by *L. leonurus* (1.12 mg/ml) yielded the highest average concentration of plant material extracted from the different extraction solvents (Table 5). The increased plant concentration extracted may be ascribed to the fine plant particles (increased surface area) of these plants presented for extraction with the different solvents. This correlates with observations of Eloff (1998b), that finely processed plant material (increased surface area) facilitates the production of concentrated plant material from extraction solvents.

Besides the different plant characteristics, extraction solvents played a major role in the concentration of plant material extracted from the medicinal plants. Previous studies have reported on the influence extraction solvents have on the concentration of plant material extracted and subsequent antimicrobial testing (Vlachos *et al.*, 1996; Martini & Eloff, 1998; Lin *et al.*, 1999).

A difference was observed in the amount of plant material extracted with the different extraction solvents and medicinal plants (Table 5). The characteristics of extraction solvents and the specific chemical composition of medicinal plants may have influenced the amount of plant material extracted.

In this study, acetone extracted the highest concentration of plant material on average, for all the medicinal plants investigated (Table 5). This finding is supported by Martini & Eloff (1998) that reported on acetone that extracted the most complex mixture of different compounds. The aqueous extraction of medicinal plants produced the lowest plant extract concentration yield in comparison to the other extraction solvents (Table 5).

Table 5 displays a slight margin (0.06 mg/ml) of difference in the average plant extracted with the acetone and methanol extraction solvents. Table 5 and Figure 8 displays the differences in plant extract concentrations obtained from the specific medicinal plants and extraction solvents, respectively. The acetone extract of *M.*

major was the highest plant extract concentration and the aqueous extract of *B. frutescens* of 0.43 mg/ml was the lowest plant extract concentration.

The prevalence of bacteria causing burn wound infections (Mayhall, 2003), initiated investigation into the antibacterial activity of medicinal plant extracts. The clinical bacterial isolates obtained from burn patients displayed their own set of antibiotic sensitivity patterns (Table's 8a & 8b), tested by the National Health Laboratory Services (NHLS).

Two antibacterial assays were used for screening the medicinal plants and determining the MIC of the plant extracts against the different bacteria. The microtitre plate assay was used for five of the eight bacteria and their bacterial strains and the agar dilution assay was used for the remaining three bacteria and their strains.

The agar dilution assay was more cumbersome than the microtitre plate assay in terms of obtaining the required volumes of the plant extract concentrations for incorporation into the melted agar for analysis. Although a total of 36 bacteria could be tested on one plate in the agar dilution assay, the microtitre plate assay has many advantages. The method is quick, easy and requires small volumes of the plant extract for testing (Devienne & Raddi, 2002). The microtitre plate assay contributes to the conservation of the plants because only small amounts of the plant extracts are required to test a wide range of concentrations and different bacteria simultaneously. Double the amount of plant material and more than one extraction was performed to obtain high plant extract concentrations ranging from 10 – 20 mg/ml for testing in the agar dilution assay.

In the microtitre plate assay, the methanol extract of *B. frutescens* inhibited a maximum of 8 of the 21 bacterial strains and a minimum of five bacterial strains in the aqueous extract. The extracts of *B. frutescens* did not inhibit any of the methicillin-resistant *S. aureus* and *P. aeruginosa* bacterial strains (Table 9b). The methanol and aqueous extracts of *L. leonurus* inhibited 15 bacterial strains, each and the acetone extract inhibited a maximum of 16 bacterial strains (Table 9b). The three extracts of *M. major* inhibited all 21 of the bacterial strains tested. The methanol extract of *Z.*

aethiopica inhibited a maximum of 10 bacterial strains whereas the aqueous extract inhibited the minimum of 7 bacterial strains for the *Z. aethiopica* extracts.

The results in Table 9b indicate that the methanol extract of all the plants inhibited the highest number of bacterial strains; therefore it displayed the highest antibacterial activity. Rabe & Van Staden, 1997 and Vlachos *et al.*, 1996 reported similar findings on the high antibacterial activity displayed by the methanol extract in comparison to other extracts.

The aqueous/water extracts of the plants inhibited the lowest number of bacterial strains in comparison to the other extracts for each plant (Table 9b). This compares with Shale *et al.*, 1999 that reported on the water being less effective than methanol at extracting the active compounds from plants.

The agar dilution assay was used for screening and testing the antibacterial activity of all plant extracts at concentrations ranging between 0.5 and 20 mg/ml against the bacterial strains of *P. mirabilis*, *S. pyogenes* and *E. faecalis* (Table's 12 & 13). Only the bacterial strains of *P. mirabilis* were inhibited by the extracts. The acetone and methanol extracts of *M. major* were the only extracts that displayed antibacterial activity (Table 12).

A comparative testing of different *M. major* acetone extractions obtained from freshly crushed leaves and refrigerated fresh leaves crushed the previous day displayed a significant difference in the antibacterial activity. The acetone extract obtained from fresh leaves crushed a day prior to extraction (kept refrigerated), failed to inhibit the *P. mirabilis* strains (Figure 14). The test indicated that the antibacterial activity was lost or absent after the overnight refrigeration of the freshly crushed leaves. The active compounds of *M. major* leaves may have been oxidised or undergone chemical alterations after overnight refrigeration, resulting in a loss of activity.

Besides the different plant extracts, the traditional preparations of the plants were tested for antibacterial activity as these preparations are used traditionally by the indigenous people (Van Wyk *et al.*, 1997). In the microtitre plate assay the *B. frutescens* infusion and the *Z. aethiopica* decoction inhibited 8 of the 21 bacterial strains. The *L. leonurus* and *M. major* decoctions inhibited 20 of the 21 bacterial

strains (Table 11). The difficulty associated with eradicating *P. aeruginosa* and MRSA infections due to antibiotic resistance, enhances the importance of antibacterial activity displayed in diluted traditional plant preparations (Table 11). The widespread antibacterial activity in *L. leonurus* and *M. major* scientifically supports the indigenous uses of these plants for treating infected and septic wounds.

The traditional preparations of these plants displayed no antibacterial activity in the agar dilution assay against the bacterial strains of *P. mirabilis*, *E. feacalis* and *S. pyogenes* (Table 14). Antibacterial activity of the traditional plant preparations against these bacteria may be present in more concentrated preparations.

In the agar dilution assay the different degrees of bacterial inhibition at a specific plant extract concentration could not be established. A complete absence of bacterial growth is required with the agar dilution assay in order to report bacterial growth inhibition for the different medicinal plants.

A more sensitive assay such as the microtitre plate assay may have detected the different degrees of bacterial inhibition compared to the complete absence of bacterial growth needed with the agar dilution assay in reporting inhibition. Regardless of the sensitivity of the microtitre plate assay in detecting small changes of bacterial growth at specific concentrations, it was not compatible with the three bacteria for antibacterial analysis.

The methanol extracts of *B. frutescens* and *Z. aethiopica* inhibited all the *S. aureus* strains. Although all the strains of *S. aureus* were resistant to penicillin the extracts of *L. leonurus* and *M. major* inhibited all the strains of *S. aureus*.

Despite the resistance of all MRSA strains to four antibiotics (penicillin, co-amoxicillin, cloxicillin and erythromycin) the extracts of *L. leonurus* and *M. major* inhibited all MRSA strains except MRSA strain [no. 4] (Table 9b). The strains of MRSA were not inhibited by the extracts of *B. frutescens* and *Z. aethiopica* except for MRSA strain [no. 1] that was inhibited by the extracts (aqueous & acetone) of *Z. aethiopica*.

In spite of the sensitivity of *S. pyogenes* strains to all the tested antibiotics (Table 8a) these strains were not inhibited by any of the plant extracts at any concentration in

the agar dilution assay (Table 12 & 13). The *E. feacalis* strains were all resistant to cotrimoxazole and tetracycline was also not inhibited by any of the plant extracts in the agar dilution assay. Despite the inability of the extracts of *M. major* to inhibit the bacterial strains of *S. pyogenes* and *E. feacalis*, the *P. mirabilis* strains was inhibited by acetone and methanol extracts of *M. major* with MIC's of 5 mg/ml (Table 12).

Increased extract concentrations of *M. major* of up to 20 mg/ml failed to display inhibition with the bacterial strains for these two bacteria. Streptococci and enterococci have been reported for their natural resistance and mechanisms that's able to defy various conventional antibiotics (Berkowitz, 1995). The natural resistance of these bacteria may contribute to the absence of inhibition by medicinal plant extracts in this study.

Although the strains of *P. aeruginosa* were all resistant to gentamycin (Table 8b), the extracts of *M. major* inhibited all the *P. aeruginosa* strains at varying concentrations (Table 9b). The extracts of *B. frutescens* did not inhibit any of the strains of *P. aeruginosa* (Table 9b). The extracts of *M. major* displayed a higher antibacterial activity in comparison to gentamycin, against the bacterial strains of *P. aeruginosa*.

All the strains of *A. baumannii* were resistant to cefazolin and cefuroxime and completely inhibited by the different extracts and traditional preparation of *M. major* (Table's 9b & 11). The strains of *K. pneumoniae* were all resistant to ampicillin and cotrimoxazole and completely inhibited by the extracts and traditional preparation of *M. major* (Table 9b). The low MIC's of plant extracts especially *M. major* against these bacteria that are resistant to standard antibiotics, are promising sources of potent antimicrobial sources.

The standard agar plate count technique was used to confirm the relative bacterial growth and bacterial stimulation of the bacterial suspensions in microtitre plate wells. Results indicated that the selected bacteria with relative growth percentages of more than 100% appear to be stimulated by the plant extract at the specific concentrations in the microtitre plate assay (Table 15).

S. aureus was inhibited in the microtitre plate assay with growth percentages less 100%. The inhibition of *S. aureus* strain [no. 1] was confirmed with the lower cfu/ml

(1.30×10^{11}) in the plant extract suspension in comparison to the bacteria control (1.34×10^{11}). Further testing is needed in the plant extract concentrations and the inhibition of the bacteria.

The antibacterial activity of the medicinal plant extracts and traditional preparations especially *M. major* and *L. leonurus* supports the traditional uses of these plants for treating infected skin lesions.

The aim of this study was achieved by proving the *in vitro* antibacterial activities of the indigenous plants used traditionally for bacterial wound infections. The objectives of screening indigenous plants for antibacterial activity and reporting on the MIC of the plant extracts were also achieved. This study may contribute to the increased scientific investigation done on indigenous medicinal plants used traditionally in the different parts of South Africa.

The different chemical composition of medicinal plants and extraction solvent characteristics influenced the amount of plant material extracted from medicinal plants. In this study *M. major* yielded the highest average concentration of plant material extracted and *B. frutescence* yielded the lowest concentration of plant material following the different extractions. The difference in the plant concentration yield for the two plants may be ascribed to the plant processing and surface area of plant material presented for extraction.

The acetone extraction solvent yielded the highest average concentration of plant material extracted and the aqueous solvent produced the lowest average concentration of plant material extracted from medicinal plants. Similar findings were displayed in previous investigations using these solvents for plant extraction (Martini & Eloff, 1998).

Consequently the acetone extract of *M. major* yielded the highest concentration of plant material extracted in comparison to the other solvents and medicinal plants.

The plant extracts of *M. major* followed by *L. leonurus* gave promising antibacterial activity against a wide range of the bacteria that was tested. Purification of the *B. frutescens* and *Z. aethiopica* plant extracts may yield more potent extracts with higher antibacterial activity.

Further testing on the plant extracts could be done on anti-inflammatory activity, antifungal activity, toxicity studies, anti-ageing effects and its effect on keratinocytes of the skin to assess the full spectrum of the skin and the benefits of these plant extracts. Some medicinal plants such as *Centella asiatica* have wound-healing properties and assist in retracting the wound by facilitating the development of normal connective tissue.

Medicinal plants used for treating burn wounds, abscesses and boils, may contain analgesic activity that assist in alleviating the pain (Van Wyk & Gericke, 2000). In vivo testing of the different plant extracts done on rats may be helpful in assessing the wound healing properties of the plants such as increasing the tissue keratinisation. The high antibacterial activity against a wide range of pathogens in this study makes *M. major* a promising plant for further investigation into the wound-healing properties and the above-mentioned studies.

The medicinal plants selected in this study may have additional properties that are beneficial to the skin when treating wound infections such as wound-healing properties and pain relief. The ethnobotanical approach to selecting plants for scientific investigation is substantiated as the traditional uses of these plants in treating bacterial wound infections are justified.

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