IMPACT OF VARIOUS BOILING INTERVALS ON THE ANTIMICROBIAL EFFICACY AND PHYTOCHEMICAL PROFILE OF SELECTED CRUDE AQUEOUS PLANT EXTRACTS, USED BY BAPEDI TRADITIONAL HEALERS IN THE TREATMENT OF SEXUALLY TRANSMITTED INFECTIONS

by

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A thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BOTANY

in the

FACULTY OF SCIENCE AND AGRICULTURE

School of Molecular and Life Sciences

at the

UNIVERSITY OF LIMPOPO

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2014

DECLARATION

The experimental work described in this thesis was conducted at the Department of Plant Science, University of Pretoria, under the supervision of Doctor T.E. Tsikhalange (Antimicrobial aspects), the Biotechnology Unit, School of Molecular and Life Sciences at the University of Limpopo, under the supervision of Professor I. Ncube (High Performance Liquid Chromatography Analysis), and the preliminary phytochemical assessment of *Aloe marlothii* in the Department of Microbiology, Biochemistry and Biotechnology, School of Molecular and Life Sciences at the University of Limpopo, under the supervision of Professor P. Masoko. All research activities were conducted from January to September 2013. This thesis, submitted for the degree of Doctor of Philosophy in Botany, in the Faculty of Science and Agriculture of the University of Limpopo, Mankweng, represents original research conducted by the author; exceptions are where the work of others is duly acknowledged in the text. Although the majority of these studies have been submitted (2013) for publication in peer reviewed journals they have not otherwise been submitted in any form for any other degree or diploma at any other University.

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To my sons Luan, Janco and Ricardo, my joy and inspiration.

ACKNOWLEDGEMENTS

My supervisor, Professor M.J. Potgieter, to whom I'm indebted for the patience and wisdom with which he guided my discovery and appreciation of the value and contribution of human physiology within the wonderful field of phytomedicine.

The completion of this thesis was a tremendous logistical challenge, and I would like to thank the following people who ensured a relatively stable research environment:

Doctor T.E. Tshikalange, my co-supervisor (UP), and Professors I. Ncube and P. Masosko (UL) who taught me so much about the various analytical procedures employed in phytomedicine. Thank you for availing your time, expertise and laboratory facilities.

Colleagues in the Faculty of Science and Agriculture; especially our Executive Dean Professor H.J. Siweya, a gentleman and leader, who made so many things possible, including the study leave that made this dream a reality. Professor P.A.S. Olivier for his encouragement and support. My Head of Department, Doctor M. van Staden who kept me informed of departemental developments in my absence.

I'm especially grateful to Professor W.J. Luus-Powell, for her dedication in taking care of the postgraduate students under our supervision, during my study leave.

To the many colleagues who showed a keen interest in my progress. You have been a constant source of support and laughter.

This research was supported by funds provided by the Departments of Physiology and Environmental Health and Biodiversity (UL), and the Department of Plant Science (UP).

ACKNOWLEDGEMENTS

I wish to express my gratitude to my wife Elana and my three amazing sons Luan, Janco and Ricardo for being my pillar of strength and for their love, inspiration and wisdom.

I thank the Almighty God for being with me and blessing me abundantly with friends who carried me in prayer during my studies.

ABSTRACT

Ethnobotanical studies are currently a major contributor to the identification of plant species used in the treatment of a large assortment of diseases, including but not limited to sexually transmitted infections. Some of these studies are comprehensive enough that detail regarding the traditional health care practice is captured; especially information related to the combination of different plant species or plant parts in the preparation of remedies, as well as preparation procedures employed. However, relevant information is often lost because a number of studies, due to poor planning and execution do not cover these aspects.

It is true that large variations exist regarding these preparation procedures. Such an example includes the preference of different boiling intervals when preparing an extract. Scientific sources highlight the fact that these variations are often regulated by cultural norms, but can also include healer specific preferences. In the process of scientifically validating the use of such extracts, innovative approaches are required. The current study is an example of such an effort, where it was noted that the Bapedi traditional healers in the Limpopo Province of South Africa, used boiling periods ranging from 5 to 20 minutes when they prepare aqueous extracts from the plant species (*Aloe marlothii*, *Catharanthus roseus, Hypoxis hemerocallidea*, *Tribulus terrestris* and *Ziziphus mucronata*) selected for this study.

Fresh plant material was collected and added to a pot of boiling tap water. At 5 minute intervals 500 ml was removed to a maximum of 20 minutes, starting with time zero, without topping-up the volume in the pot. The aim was to collect the highest possible yield, and to subsequently subject the freeze-dried particulates to antimicrobial assays (micro-dilution), using a 50 mg/ml stock solution. Pathogens included in this study were: *Candida albicans*, *Neisseria gonorrhea*, *Proteus vulgaris* and *Staphylococcus aureus*. A small volume (2 ml) of each crude aqueous extract was also assessed via High Performance Liqiud Chromatography, to determine fluctuations in alkaloid, phenolic and terpenoid levels.

Aloe marlothii leaf and root extracts exhibited a wide range (MIC 0.09–12.5 mg/ml) of inhibitory activities against the above mentioned pathogens. Results

indicated that this plant species is not only underutilized from a research perspective, its therapeutic value is completely underestimated. The fact that its combined root and leave aqueous extract had an minimum inhibitory concentration (MIC) <0.09 mg/ml at time zero should focus attention on further phytopharmaceutical analyses, to establish its potential value in future drug development.

Ziziphus mucronata, in combination with either *H. hemerocallidea* or *T. terrestris*, exhibited very poor antimicrobial activity. The fact that *Z. mucronata* featured in previous research as a common denominator in a number of multi-plant extracts, created the impression that this species might play a role in the possible antimicrobial activity of these crude extracts. This theory could not be confirmed in our study; indicating that the inclusion of this species might be related to a non-pathogenic function.

Catharanthus roseus, a species exclusively used to treat gonorrhea, showed potential in the inhibition of *N. gonorrhea*, with all MICs recorded at 0.78 mg/ml. This level of activity does not fall within the excellent or even clinically relevant range, yet its continued traditional use supports the notion that its therapeutic impact might involve far more than only antibacterial capabilities.

High performance liquid chromatography results established the presence of time-dependent extraction relationships for alkaloids, phenolics and terpenoids. Combining this data with the antimicrobial results highlighted the following. Terpenoids present in *A. marlothii* and *Z. mucronata* / *T. terrestris* extracts were implicated in its antigonococcal efficacy. This was different from *C. roseus*, where alkaloids were identified as the possible source of antigonococcal activity. *Proteus vulgaris* showed a particular sensitivity for alkaloids and terpenoids present in *A. marlothii* extracts. *Candida albicans* displayed a diverse reaction when exposed to the various crude extracts. Alkaloids present in *A. marlothii* root and leaf extracts, and terpenoids in the *A. marlothii* leave / root and *Z. mucronata* / *H. hemerocallidea* combinations showed potential as anti-fungal compounds.

This study found that some plant species require an extended boiling period to exhibit antimicrobial activity; however, a few of the species showed this potential with limited boiling. Current findings partially support the use of some of these species, such as *C. roseus* and *A. marlothii* subsp. *marlothii*, in the treatment of STIs. The

fact that very poor antimicrobial activity was detected for a number of these extracts, and that these plants species remain highly used in traditional medicine, opens up the possibility that they might contribute to the alleviation of symptoms via a non-pathogen dependent mechanism.

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ACE	Acetylcholine esterase
AIDS	Acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
BEA	Benzene / ethanol / ammonia hydroxide
BPH	Benign prostatic hyperplasia
CDC	Center for Disease Control
CEF	Chloroform / ethyl acetate / formic acid
COX	Cyclooxygenase
CVD	Cardiovascular disease
DCM	Di-chloro-methane
DHEA	Dehydroepiandrosterone
DM	Diabetes mellitus
ED	Erectile dysfunction
EMW	Ethyl acetate / methanol / water
EtOH	Ethanol
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
INT	P-iodonitrotetrazolium violet
LDL	Low density lipoprotein
LH	Luteinizing hormone
LOX	Lipoxygenase

List of Abbreviations

MH broth	Mueller-Hinton broth
MIC	Minimum inhibition concentration
NO	Nitric oxide
PID	Pelvic inflammatory disease
RSA	Republic of South Africa
STI	Sexually transmitted infections
TLC	Thin-layer liquid chromatography
WHO	World Health Organization

“Research designed for the benefit of local communities, planned and implemented together with these communities, so as to focus on local health priorities and design locally acceptable and achievable solutions, can boost the impact of scientific research on primary healthcare and uphold the potential to achieve true social value”

Ina Vandebroek, Journal of Ethnopharmacology, 2013.

PROLOGUE

GENERAL INTRODUCTION

Annually more than 340 million cases of curable sexually transmitted infections are reported globally; among adults aged 15–49. According to the World Health Organization there are approximately 30 sexually transmissible parasitic pathogens (WHO, 2007). However, only a small number of pathogens such as *Chlamydia trachomatis*, *Neisseria gonorrhea* and *Treponema pallidum*, are responsible for most of the disease burden. Currently, the primary concern regarding bacterial pathogens relate to their increased resistance against known antibiotics. Thus, increasing the risk of suffering detrimental effects as effective therapeutics are either not available, very limited or ineffective. Against this backdrop, there has been a focused approach to identify new antibiotics to address the effective treatment of infective diseases.

This approach redirected the attitude towards medicinal plants used traditionally to treat bacterial infections. It is also true that the floral diversity makes it virtually impossible to assess all plant species for their possible antimicrobial capacity. Therefore, it makes sense that traditional medicine can significantly contribute to the identification of species that shows potential in the treatment of microbial infections. Various crude plant extracts have shown bactericidal and fungicidal potential; however, Gram-negative bacteria still presents a significant challenge as they tend to be fastidious and exhibit low susceptibility to antibiotics.

PROLOGUE

CHAPTER 1

1.1 GENERAL LITERATURE REVIEW

1.1.1 Traditional healers

Traditional healers are known by many names, depending on the cultural or ethnic groups involved. However, for the purpose of this work the most relevant approach would be to view them in their capacity as primary health care providers. Within the South African context, this is an important aspect as current estimates are that between 60–80% of people would rather consult a traditional healer, before considering the services of modern allopathic medicine (Setswe, 1999).

In South Africa, in an effort to protect traditional healers and to regulate their practices, the Traditional Health Practitioners Act (Act 35 of 2004) was adopted (Republic of South Africa, 2004). According to this act, the focus is on the performance of a function, activity, process or service that is based on the traditional philosophy which, amongst others, includes the use of traditional medicine. This act clearly excludes the participation of individuals who practices other professions as endorsed by acts such as the Nursing Act, Allied Health Professions Act and the Pharmacy Act; or any other activities which are not based on traditional philosophy.

Currently, a dual approach exists as to the definition of traditional medicine. Firstly the local definition as depicted in Act 35 of 2004 (Republic of South Africa, 2004), where it is described as:"… an object or substance used in traditional health practice for (i) the diagnosis, treatment or prevention of a physical or mental illness; or (ii) any curative or therapeutic purpose, including the maintenance or restoration of physical or mental health or well-being in human beings". However, the requirements of this act also stipulate that it should exclude any dependence-producing or dangerous substances, or drugs. The second definition pertaining traditional medicine is the approach of the WHO, who refers to it as "African traditional medicine". Their Centre for Health Development describes it as follows (Richter, 2003): "The sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or societal

imbalance, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing". Clearly from these definitions traditional medicine, whether African or South African involve far more than just the use of herbal remedies. In essence it represents a holistically intertwined hierarchy regulated by cultural and religious beliefs.

It is well established that Africa, and more so South Africa, has an extended and very rich history regarding the traditional use of medicinal plants. Not surprisingly the diversity of plants used therapeutically often varies within the confines of local traditions, cultures and customs (Watt and Breyer-Brandwijk, 1962).

1.1.2 Phytomedicine

Per definition phytomedicine refers to herbal preparations produced by subjecting plant materials to extraction, fractionation, purification, concentration or other physical or biological processes (WHO, 2001). Evidence support the fact that first-generation medicinal plants were used as crude extracts; these plants were solely selected on the basis of empirical evidence. Second-generation phytopharmaceutical agents differed significantly from the crude extracts as they were pure molecules; but when compared to synthetic therapeutic agents the only difference was their origin (Iwu et al., 1999). Third-generation plant medicines face a completely different challenge; their formulation is based on well-controlled clinical and toxicological studies, and in the final stage the emphasis is on improvement of quality, efficacy, stability and safety of such preparations (Petrovic et al., 1999).

1.2 SEXUALLY TRANSMITTED INFECTIONS

1.2.1 General introduction

In many developing countries, including South Africa, sexually transmitted infections (STIs) are notable contributors to disease burden (Rowley and Berkley, 1998). In the early 1990s the WHO, in the light of its concern regarding the difficulties associated with the treatment of STIs in developing countries, instituted syndromic management

guidelines aimed at the effective treatment of STIs and other genitourinary tract infections (WHO, 1991). This strategy, quite similar to that currently employed by some South African ethnic groups (De Wet et al., 2012; Erasmus et al., 2012), aims to treat all STI patients aIcording to their symptoms rather than postponing treatment until laboratory confirmation has been received.

Interventions such as this are commendable, yet current sexual health challenges should also be considering the impact of asymptomatic infections; particularly more prevalent bacterial infections such as chlamydia, gonorrhea and syphilis. Asymptomatic individuals in especially rural settings, due to the lack of modern screening facilities, are oblivious to the fact that they are infected and can still spread the disease. Therefore, focusing on symptomatic management is a worthwhile, albeit not optimal, approach to addressing the disease burden. Traditional healers, even with the lack of modern laboratory facilities, can significantly contribute towards the symptomatic management of STIs.

1.2.2 *Neisseria gonorrhea*

1.2.2.1 General description

Gonorrhea is a transitional infection of columnar epithelium caused by the Gram-negative diplococcus *Neisseria gonorrhea* Zopf (Bokaeian et al., 2011). The resourceful nature of the gonococcus, and its capacity to deal with changing conditions, was first reported after the introduction of sulfonamides; the first efficient therapeutic agents (Dunlop, 1949). Unfortunately, the inappropriate use of antibiotics has contributed significantly to the antibiotic resistance of *N. gonorrhea* (Ye et al., 2002); thus resulting in enormous resistance to antibiotics formerly used effectively in the treatment of gonorrhea (Zheng et al., 2003). It is believed that its resistance to antimicrobials is either due to chromosomal mutations or the acquisition of R plasmids (Willcox, 1970).

The bacterial membrane component, Pili and Opacity- associated proteins (Opa), are vital to establish the initial contact and adherence to its epithelial host cell (Merz and So, 2000). Subsequent to this, the bacterium must penetrate the cell membrane of the host cell, in order to invade it. Even though various mechanisms for

this trans-membrane movement exist, the most reasonable ones include: (i) endocytosis of the bacterium by the male urethral cells, as well as cells derived from the cervix and oviducts; suggesting transcytosis (Ilver et al., 1998; Morales et al., 2006), or (ii) that the gonococci cross the epithelial barrier, in the male urethra, to the subepithelial tissue via the paracellular spaces (Harkness, 1948).

1.2.2.2 Epidemiology

The primary route for exposure to *N. gonorrhea* is sexual intercourse. This bacterium flourishes in the moist environment of the urogenital tract. It is therefore not surprising that the cervix, vagina, urethra, uterus and oviducts are important target areas of infection (RodrÍguez-Tirado et al., 2012), thereby supporting the fact that this organism is one of the most common causes of female infertility (Dudas and Hardin, 2000; Weinstock et al., 2004). The clinical features, including signs and symptoms, when present, depend largely on the site of infection (Sherrard, 2010).

Approximately 75% of females who acquire gonorrhea are asymptomatic (Jones and Lopez, 2006). In those who present with symptoms, the most common signs are a mucopurulent cervicitis and vaginal discharge, which may be caused by concomitant infections with other pathogens (Sherrard, 2010). The first sign is usually the presence of a clear or whitish fluid discharge from the vagina. This discharge shortly changes to a yellowish or even greenish colour, hence the reference to a purulent discharge, which actually indicates a pus-filled discharge (Jones and Lopez, 2006). It is noteworthy that in females with cervical infections, the vaginal discharge, low abdominal pain or pelvic pain is nonspecific (Sherrard, 2010).

If such an infection is left untreated, either because of its asymptomatic presentation or ignorance, it can pose a threat to the reproductive health of the individual. The bacteria can infect the uterus, fallopian tubes and other pelvic and abdominal organs, resulting in pelvic inflammatory disease (PID). It can also initiate inflammation of the heart, brain, meninges, eyes, skin and joints. It is also possible to infect the fetus *in utero*, thereby increasing the risk for spontaneous abortion in the first trimester (Jones and Lopez, 2006).

Neisseria gonorrhea is an established sexually transmitted pathogen implicated in the aetiology of urethritis (Ishihara et al., 2004; Sturm et al., 2004). In South Africa,

the most common clinical condition urethritis (Pham-Kanter et al., 1996) includes symptoms such as a urethral discharge with/without a burning sensation on micturition and occasionally dysuria (De Jongh et al., 2009; Sherrard, 2010). Unlike females, most males (70–90%) develop recognizable symptoms of gonorrhea (Jones and Lopez, 2006). In general, males with urethral infection usually develop symptoms 3–10 days post exposure (Sherrard, 2010); however, it may take up to 30 days for the symptoms of infection to appear (Jones and Lopez, 2006). If left untreated, symptoms resolve in most cases, though, these males can remain infectious for many months. It is interesting to note that the urethral discharge of gonorrhea has no pathognomonic features (Sherrard, 2010).

1.2.2.3 Traditional medicine

1.2.2.3.1 Diagnosis

In traditional health care, the diagnosis of an STI is predominantly based on the presentation of symptoms, as laboratory facilities are either not available or too expensive for confirmation of an infection. De Wet et al. (2012), put the challenges regarding the diagnosis of a specific STI in context when they reported that interviewees knew the symptoms caused by STIs without knowledge regarding the specific STI that they were treating. Gonorrhea was identified by the presence of an abnormal urethral discharge, also referred to as “drop”. Similarly the presence of external sores (De Wet et al., 2012), reflects on the ulcerative nature of some STIs, including, but not limited, to gonorrhea (Jones and Lopez, 2006).

1.2.2.3.2 Treatment

Various plant species are used as herbal remedies in the treatment of gonorrhea. Some of these have been validated, via phytochemical and biochemical analysis, to exhibit antimicrobial activity.

Leaf infusions or juice are often used in the treatment of gonorrhea. Amusan et al. (2007) reported the use of a leaf infusion prepared from *Acrotome hispida* Benth. Juice from the leaves of *Aloe ferox* Mill. is also utilized (Watt and Breyer-Brandwijk,

1962). Similarly Turner (2001), reported the use of another *Aloe* spp., *viz A. marlothii* subsp. *marlothii* to treat unspecified STIs.

The use of corms and leaves in multiple-plant herbal remedies used to treat gonorrhea, have been reported. It was indicated that *H. hemerocallidea* is often used in combination with species such as *Senecio serratuloides* DC., *Tabernaemontana elegans* Stapf, *A. marlothii*, *Carica papaya* L., *Euphorbia tirucalli* L. and *Ozoroa engleri* R.Fern. & A.Fern. (as cited by De Wet et al., 2012).

Neisseria gonorrhea is treated with *Cussonia* spp., specifically *C. arborea* where either an aqueous decoction or maceration of root or bark is topically applied. The consumption of a root tea or bathing in a vapor bath made of the root is also employed (Watt and Breyer-Brandwijk, 1962; Ndubani and Höjer, 1999). Coincidentally, De Villiers et al. (2010), reported antigonococcal activity (*N. gonorrhea* ATCC 19424) with a minimum inhibition concentration (MIC) ranging from 0.02–0.7 mg/ml for the *Cussonia* spp. they tested. Using the same bacterial strain (ATCC 19424), Van Vuuren and Naidoo (2010) reported significant bactericidal activity for methanol:dichloromethane (1:1) extracts from four species; *Hypericum aethiopicum* Thunb. subsp. *aetihiopicum* (0.3 mg/ml), *Tarchonanthus camphoratus* L. (0.5 mg/ml), *Terminalia sericea* Burch. ex DC. (1.0 mg/ml), and *Croton gratissimus* Burch. var *gratissimus* (1.0 mg/ml). It is interesting that methanol:dichloromethane extracts prepared from the leaves of *Catharanthus roseus* had insignificant activity (4.0 mg/ml). Interest in this species is based on its exclusive use by the Bapedi to treat gonorrhea, with the exception being that they use the roots and not the leaves (Semenya, 2012).

1.2.3 *Proteus vulgaris*

1.2.3.1 General description

Proteeae are widespread in the environment, and constitutes part of the normal flora found in the human digestive tract. The genus *Proteus* currently consists of a number of species, including *P. vulgaris*. The term *Proteus* means “changeability of form, as personified in the Homeric poems in Proteus… and has the gift of endless transformation” (Wenner and Rettger, 1919). Since its original description by Hauser

(1885), various taxonomic studies have appeared to address the intricacies of this genus (Table 1.1).

Table 1.1: Timeline of the genus *Proteus*.

Date	Event	Authors
1885	Original description of genus *Proteus*, including species *P. mirabilis*, *P. vulgaris*, and *P. zenkeri*	Hauser
1919	Species separation for *P. mirabilis* and *P. vulgaris*, based on sugar fermentations	Wenner and Rettger
1966	First description of *P. myxofaciens*	Cosenza and Podgwaite
1978	*P. vulgaris* biogroup 1 defined	Brenner et al.
1982	First description of *P. penneri*, as well as the establishment of two additional biogroups of *P. vulgaris*	Hickman et al.
1993	Separation of *P. vulgaris* biogroup 2 and further subdivision of biogroup 3 into two separate "taxa", with the use of SDS-PAGE	Costas et al.
1995	Replacement request for *P. vulgaris* type strain NCTC 4175 with ATCC 19905	Brenner et al.
1999	Replacement of *P. vulgaris* type strain with ATCC 29905 granted	Trüper

Adapted from: O'Hara et al. (2000a).

1.2.3.2 Epidemiology

Gram-negative bacteria, such as those from the genus *Proteus*, has the ability to cause nosocomial, wound and urinary tract infections (UTIs). Although the genus is frequently implicated as a causative agent in the development of UTIs (Kippax, 1957; Mishra et al., 2013), it is seldom considered to be a nosocomial pathogen *per se* (O'Hara et al., 2000a). Urinary tract infections are one of the more common manifestations of bacterial infections, and a gender disparity indicates that females are more affected than males; and that age is a compounding factor (Jarvis and Martone, 1992; Williams and Schaeffer, 2004).

In the reproductive tract, UTIs involving the lower parts of the tract normally includes infection of the bladder (cystitis); whereas infections in the upper parts involve the kidneys (pyelonephritis). It is possible that the presentation of the disease

may vary from a fairly harmless cystitis to severe sepsis which is often fatal (Tal et al., 2005). It is also possible, specifically in males, that genitourinary infections can contribute to Fournier's gangrene; a synergistic polymicrobial necrotizing fascitis of the penis, scrotum and perineum (Korkut et al., 2003). As a matter of fact, in its pathogenesis as well as organisms involved, both *P. vulgaris* and *P. mirabilis* have been implicated in this (Morua et al., 2009); clearly illustrating the debilitating impact of this pathogen.

1.2.3.3 Traditional medicine

1.2.3.3.1 Diagnosis

The traditional diagnosis once again, even if premature and shortsighted in nature, is based on the presence of symptoms. More specifically the presence of an abnormal, foul smelling, urethral discharge (Erasmus et al., 2012). Even though infection with *P. vulgaris* is quite capable of presenting this symptom; implicating this pathogen in the etiology is speculative at best. Currently traditional medicine does not employ modern scientific methods to positively identify the causative agent; as it is more concerned about alleviating symptoms than the origin of the infection.

1.2.3.3.2 Treatment

No ethnobotanical data exist, supporting the use of medicinal plants to explicitly treat *P. vulgaris*. However, that being said, various studies (Abraham and Thomas, 2012; Deng et al., 2012; Yildirim et al., 2012; Dubey and Padhy, 2013; Jaberian et al., 2013) have employed this pathogen in their test protocols, to assess the antimicrobial efficacy of plant extracts.

1.2.4 *Candida albicans*

1.2.4.1 General description

The genus *Candida* falls within the class Deuteromycetes; a class previously described as a "taxonomic pit" into which yeasts in general but also those exhibiting

other exceptional phenotypic characteristics have been thrown (Odds, 1987). Currently approximately 200 biologically diverse species are recognized in this class.

The genus *Candida* incorporates characteristically white asporogenous yeasts, which are capable of forming pseudohyphae. Species are primarily characterized according to colonial morphology, carbon utilization and fermentation (Shepherd et al., 1985).

The cell wall of *C. albicans*, a dimorphic fungus, is primarily composed of three polysaccharides; mannan, glucan and chitin. There is a large variation in the number of layers as well as their morphology; and it is believed that this diversification is related to factors such as the stage of growth, growth form and the culture medium used (Garzon et al., 1989).

1.2.4.2 Epidemiology

There are seven clinically relevant *Candida* spp., with *C. albicans* the most frequently isolated and virulent in humans (Samaranayake and Macfarlane, 1990). A considerable range of human infections are caused by *C. albicans* and other related species. These infections can range from relatively insignificant conditions such as genital and oral thrush to fatal, systemic co-infections in immune-compromised patients (McCullough et al., 1996; Sardari et al., 2000; Shai et al., 2008). It has been hypothesized that most individuals usually carry a single *Candida* strain; however, it has been shown that infection with multiple *Candida* spp. is possible (Odds, 1987).

It is generally accepted that the digestive tract is the major domain of commensal *Candida* spp. It was found that if *C. albicans* are present in the digestive tract, in high enough numbers, that it can easily spread from this site and cause fungaemia and funguria (Krause et al., 1969). Host tissue mucosal surfaces are the primary target of pathogenic *Candida* spp., and as such successful colonization and infection depends on its ability to adhere to such surfaces. This is supported by the fact that the more commonly presenting species (*C. albicans* and *C. tropicalis*) are known to adhere to host cells to a greater extent than nonpathogenic species (*C. krusei* and *C. guilliermondii*) (McCullough et al., 1996).

1.2.4.3 Traditional medicine

1.2.4.3.1 Diagnosis

Some ethnobotanical evidence exists supporting the use of plant extracts in the treatment of *Candida* infections. Motsei et al (2003) and Runyoro et al. (2006) reflected on traditional uses without elaborating on the symptoms used in the diagnosis of *C. albicans* infections. These pathogens are known for their impact on mucosal membranes; it is therefore reasonable to argue that the presence of an abnormal urethral or vaginal discharge would be used in the diagnosis.

1.2.4.3.2 Treatment

Treatment protocols, in general, entails the use of various plant parts to prepare aqueous extracts or poultices. The extracts are consumed orally, and either taken twice or thrice daily. The use of poultices relates to its application to the infected site, which can be either the genitals or the mouth or both (Hutchings et al., 1996; Motsei et al., 2003; Runyoro et al., 2006).

1.3 MEDICINAL PLANTS USED IN THIS STUDY

Five plant species have been selected for this study. Their selection was based on previously published work (Erasmus et al., 2012; Semenya, 2012) that highlighting their traditional medicinal value, among the Bapedi, in the treatment of STIs.

These species include: the tree aloe *Aloe marlothii* A. Berger subsp. *marlothii*, the exotic species *Catharanthus roseus* (L.) G.Don, the African potato *Hypoxis hemerocallidea* Fisch.Mey. & Ave-Lall., *Tribulus terrestris* L. and *Ziziphus mucronata* Willd. subsp. *mucronata*.

Aloe marlothii A.Berger subsp. *marlothii*

ASPHODELACEAE

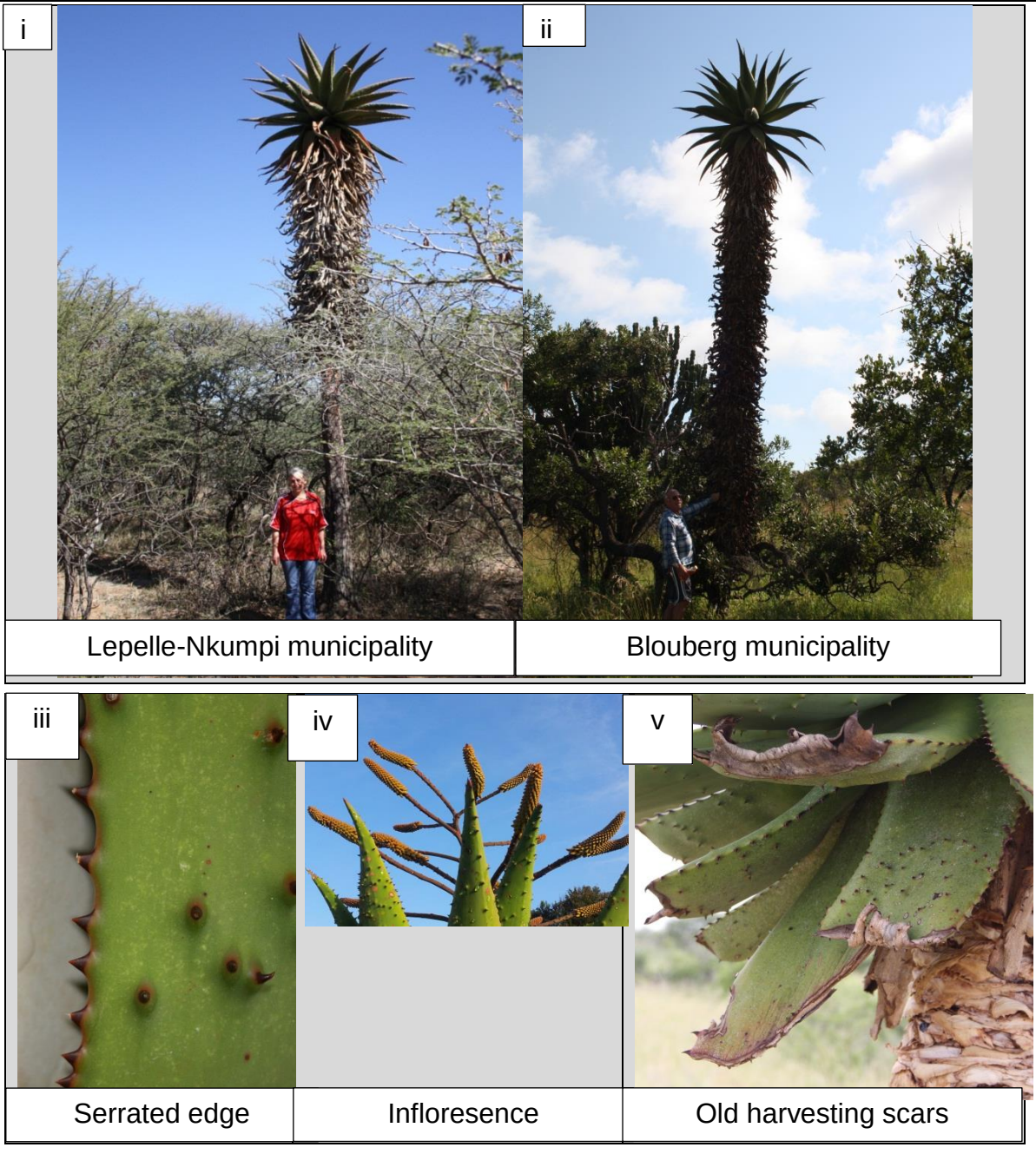

Figure 2.1: *Aloe marlothii* subsp. *marlothii*.

Mountain aloe (English); Bergaalwyn (Afrikaans); Sekgophana (North Sotho).

CHAPTER 2: *ALOE MARLOTHII*

2.1 BOTANICAL DESCRIPTION

Aloe marlothii ranges from 2–10 m (Fig. 2.1 (i) and (ii)), with a single bearded stem. The bearded appearance is created by the presence of old, dry leaves covering a significant section of the stem, especially in younger plants.

Approximately 40–50 dull grey-green leaves form a dense rosette; edged with reddish brown teeth and with scattered reddish brown spines covering the surface (Fig. 2.1 (iii)). The leaves are arcuate-incurved to spreading or slightly recurved with increased age. They are D-shaped with the following dimensions; 75–150 X 7.5–25 cm.

Flowerheads present with up to 30 branches; bracts ovate-acute, 4–9 X 2–5 mm, 3–5 nerved. The flower spikes are 30–50 cm long, and are characteristically borne horizontally (Fig. 2.1 (iv)).

Flowers are yellow to red in bud, and yellow to orange at flowering. They are cylindric to ventricose (22–25 mm long); with outer segments that connate for one third to half of their length; and the inner segments adnate to outer in basal third. Pedicles are normally 3–5 mm in length.

2.2 HABITAT AND DISTRIBUTION

It is widespread and conspicuous, where it grows in warm valleys and hill slopes (Cousins and Witkowski, 2012). There is an inclination to grow on rocky hills; however, it is often noted in the bush, where similarly to the rocky hills they frequently form extensive stands (Bredenkamp and Van Vuuren, 1987).

2.3 USES

2.3.1 Plant parts

Various parts of *Aloe* spp. are used for medicinal purposes. Existing literature supports the overwhelming use of leaf infusions (Roberts, 1990), poultices (Kambizi and Afolayan, 2001), extracts and decoctions (Amusan et al., 2007). Very little documented proof exists regarding the use of other parts. However, in Uganda root bark is used (Lamorde et al., 2010), and in South Africa the roots are used in combination with leaves (Semenya, 2012).

2.3.2 Therapeutic and prophylactic application of *Aloe marlothii*

The focus of this, and other similar, topics reflects on therapeutic application which demonstrates the healing (medicinal) or palliative properties of a species or compound. Palliative indicating the ability to treat some symptoms, without primarily focusing on the healing of the patient; e.g. treatment of pain in a person with terminal cancer. Prophylactic would then focus on the ability of any species or agent to prevent a diseased state.

The genus *Aloe* is widely used to treat various ailments. There is extensive scientific support for the various bio-active compounds, isolated from *Aloe* spp., and their prophylactic and therapeutic application in the treatment of various ailments. As a laxative in the treatment of constipation, Steenkamp and Stewart (2007) attributed the pharmacological effect to the presence of anthraquinone, particularly aloe-emodin.

Chronic diseases of life style, such as cardiovascular disorders and diabetes mellitus (DM) are often treated with remedies prepared from *Aloe* spp. Recently, Semenya et al. (2012a) reported the use of *A. marlothii* among the Bapedi in the Limpopo Province, to treat DM. In Swaziland, leaf extracts of *A. arborescens* Mill. are also used to treat DM (Amusan et al., 2007). These findings are well supported by the fact that *Aloe* spp. are well known for their hypoglaecemic effects (Gurib-Fakim, 2006). Cardiovascular health likewise benefits from the traditional use of *Aloe* spp. leaf decoctions. As early as 1986, Grindlay and Reynolds reported that the use of

this genus can decrease blood lipid levels, thus reducing the risk of cholesterol-related incidents. The report on Swazi phytomedicine (Amusan et al., 2007) is somewhat vague regarding what is meant by “cardiac problems”; however, this does not change the fact that *A. marlothii* and *A. saponaria* (Aiton) Haw. are used to treat such problems. Anti-oxidant activity reflects positively on the use of bio-active compounds in the alleviation of symptoms associated with/or in the prevention of cardiovascular diseases, cancer, neuro-degeneration and diabetes. It is of interest to note that the majority of phenolic/polyphenols, indoles and alkaloids identified in *A. ferox* are known to possess anti-oxidant activity (Loots et al., 2007). In a study on *Aloe* spp. (Ajabnoor, 1990), the hypoglaecemic effect of *Aloe* and its bitter principle was thought to be mediated via the stimulation of synthesis and/or the release of insulin from the β-cells, Islets of Langerhans, in the pancreas.

Disorders related to the optimal function of the gastro-intestinal tract are often treated with herbal remedies. Various species, such as *A. ferox* Mill. (Steenkamp and Stewart, 2007) and *A. greatheadii* Schönland (Maroyi, 2011), are used to treat constipation, mainly as a result of its laxative properties. Tea prepared from chopped *Aloe marlothii* subsp. *marlothii* leaves are used for the treatment of stomach ailments (Roberts, 1990). This species is also used for the treatment of internal parasites (Watt and Breyer-Brandwijk, 1962) and malaria (Clarkson et al., 2004).

Skin disorders are quite common and often treated via the use of *Aloe* spp. The medicinal application of this genus in the treatment of skin afflictions is very diverse. *Aloe ferox* leaf extracts are used to enhance wound healing (Tang et al., 2007); chopped *A. globuligemma* Pole Evans leaves are used as a poultice to cover inflamed sores (Kambizi and Afolayan, 2001); *A. marlothii* subsp. *marlothii* leaves are used to treat general sores (Luseba and Van der Merwe, 2006); and *A. variegata* L. is used to treat callosities, bunions, boils and inflamed wounds (Van Wyk et al., 2008). Assessment of anti-inflammatory activity is usually achieved via the COX-1 and COX-2 assays. Chen et al. (2012) found that aqueous extracts of *A. ferox* exhibited anti-inflammatory and analgesic activity. Traditional healers are primarily dependent on the presence of symptoms for their diagnosis of an ailment. Among the Bapedi (Erasmus et al., 2012) an abnormal urethral discharge is indicative of an STI. This discharge can easily result from inflammation and/or infection with

pathogens such as *Neisseria gonnorhoea* and *Chlamydia trachomatis; t*herefore, the current approach to simultaneously test for anti-inflammatory and bactericidal activity. Recently Chen et al. (2012) assessed the anti-bacterial activity of *A. ferox*. It was found that a methanol extract as well as aloin exhibited activity (0.5 and 0.1 mg/ml respectively) against *N. gonorrhea*. This is similar to an earlier report by Kambizi et al. (2005) who attributed their anti-microbial activities to the presence of aloin A, but also that of aloe-emodin and chrysophanol. According to Ferro et al. (2003), the bactericidal activity of aloe-emodin relates to its ability to affect N-acetyl transferase in some bacteria, notably *Helicobacter pylori*. Of interest is the fact that McGaw et al. (2000) failed to report antibacterial activity for *A. arborescens* and *A. marlothii*. In this study hexanic, ethanolic and aqueous extracts were tested against two Gram-negative (*Escherichia coli* and *Klebsiella pneumonia*) and two Gram-postive (*Bacillus subtilis* and *Staphylococcus aureus*) bacterial strains. Even though these earlier findings reflect negatively on the anti-bacterial use of extracts from *A. marlothii*, current traditional uses include this species in the treatment of *C. trachomatis* and *N. gonorrhoea* (Erasmus et al., 2012). As Grace et al. (2008) so eloquently stated; frequently documented *Aloe* spp. warrant research into their potential as prophylactic or therapeutic agents.

Irrespective of socio-cultural orientation, reproductive health care is a priority to all individuals of reproductive age. Within this context it is not surprising to find that *Aloe* spp. plays a pivotal role in primary health care delivery. The most prominent use of this genus revolves around the treatment of STIs. Species used include *A. parvibracteata* Schönland (Bruschi et al., 2011), *A. ferox* (Grace et al., 2008), *A. barbadensis* Mill. (Halberstein, 1997), *A. globuligemma* (Kambizi and Afolayan, 2001), *A. greatheadii* (Maroyi, 2011) and *A. chabaudi* Schönland var. *chabaudi* (Mabogo, 1990).

Equally important, but not as well documented as the STIs, is the use of herbal remedies to regulate reproductive functions. A number of *Aloe* spp., such as *A. barbadensis* and *A. vera* (Halberstein, 1997) are used to regulate the menstrual cycle, mostly the duration and volume of menstrual flow. *Aloe buetnerri* A.Berger is used for the treatment of dysmenorrhea and infertility (Telefo et al., 1998). Pregnant

women use *Aloe barbadensis* as a childbirth aid, whereas *A. vera* is used as an abortifacient to terminate unwanted pregnancies (Halberstein, 1997).

Recently, York et al. (2011) reported on the use of *A. marlothii* in Maputaland, KwaZulu-Natal, South Africa. Its traditional use in the treatment of respiratory infections and related symptoms was reported. Confirming the medicinal value of the genus, and more specifically that of the species in South Africa.

2.4 PHYTOCHEMICAL CONSTITUENTS

The current literature, regarding traditional as well as scientific usage of this species, highlights the employment of both leaves and roots. Support for this trend is supplied by Van Wyk et al. (1995) who reported that the phytochemical profile of roots are completely different when compared to that of leaves. This genus (Asphodelaceae) is characterized by polysaccharides accumulating in the leaves, as well as anthranoids and anthra-glycosides. However, Asphodelaceae do not accumulate steroidal saponins (Gurib-Fakim, 2006). In *Aloe* spp. a number of anthraquinones have high medicinal value; aloesin (Fig. 2.2 (i)), aloeresin A (Fig. 2.2 (ii)) (Van Wyk et al., 2009), aloin A and B (Fig. 2.3 (i) and (ii)), aloe-emodin (Fig. 2.4 (i)), aloe-bitters and aloe-lectin (Wang, 2009). The presence of aloeresin and aloeresin A in the leaves of *A. marlothii* was confirmed via HPLC analysis (Van der Bank et al., 1995). Furthermore, leaves contain various phenolic compounds, including anthrone-C-glycosides, phenylpyrone derivatives and chromones (Fig. 2.4 (ii)) (Reynolds, 1985).

Van Wyk et al. (1995) analysed roots collected from 172 *Aloe* spp., and found that chrysophanol and asphodeline are characteristic constituents of the subterranean metabolism of *Aloe* spp. They identified these two compounds as well as the following three in the roots of *A. marlothii*; aloesaponarin I, aloesaponol I and aloesaponol II. More recently it was confirmed that anthraquinones such as chrysophanol and aloesaponarin I are indeed confined to the roots of *Aloe* spp., specifically the roots of *A. ferox* (Chen et al., 2012).

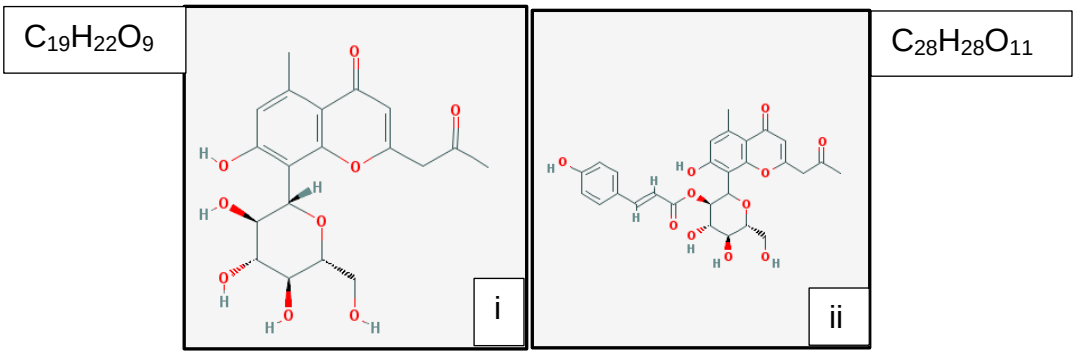

Figure 2.2: Chemical structure of Aloesin (i) and Aloeresin A (ii).

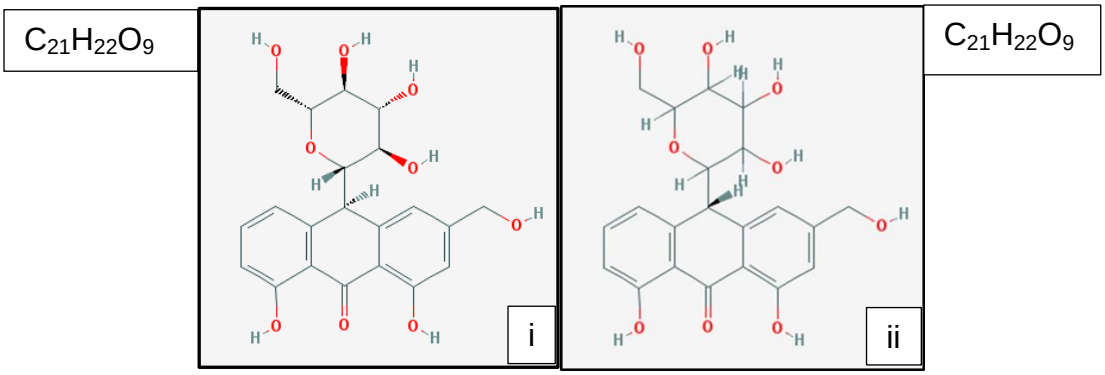

Figure 2.3: Chemical structure of Aloin A (i) and Aloin B (ii).

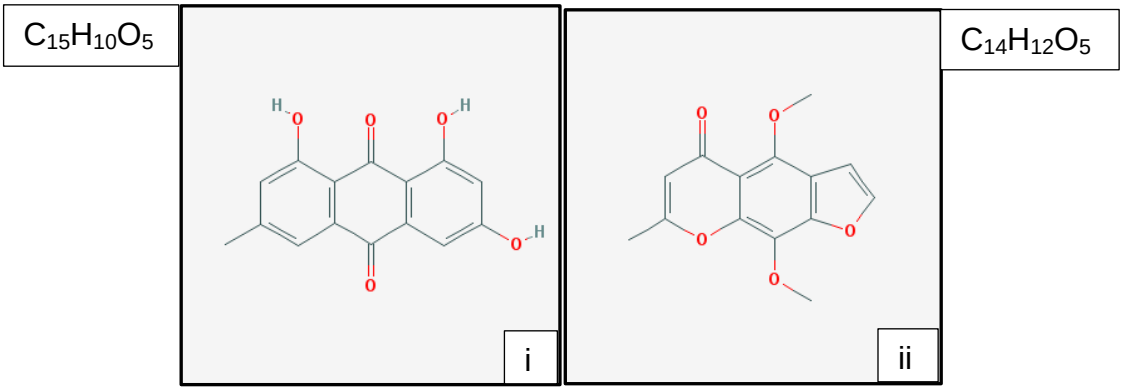

Figure 2.4: Chemical structure of Aloe emodin (i) and Chromone (ii)

Catharanthus roseus (L.) G.Don

APOCYNACEAE

i

Wild population of *Catharanthus roseus,* Mafefe, Lepelle-Nkumpi municipality

ii

Glossy, oblong leaves

iii

Typical pink and white- flower

iv

Subterranean parts

Figure 3.1: *Catharanthus roseus*.

Madagaskar periwinkle (English); Begraafplaas blom (Afrikaans); lepolomo la pinki le le drop (North Sotho).

CHAPTER 3: *CATHARANTHUS ROSEUS*

3.1 BOTANICAL DESCRIPTION

Catharanthus roseus (Fig. 3.1), an ever blooming species, is an erect perennial herb (Fig. 3.1 (i)), with a slightly woody base, that can grow up to one metre in height. It is often regarded as a weed.

Leaves are oval to oblong (Fig. 3.1 (ii)), 2.5–9 cm long and 1.0–3.5 cm wide. It is dark green and glossy, hairless and exhibits a very prominent pale-white midrib (Van Wyk et al., 2009). Leaves have a short petiole (1.0–1.8 cm), and are arranged in opposite pairs.

Flowers of this popular and attractive garden plant have five lobe-like petals and can range in colour from pink to white (Fig. 3.1 (iii)), or white with a pink centre (Chauhan et al., 2012). The pink-flowered form (Fig. 3.1 (iii)) gives a higher yield of foliage and roots (Kamboj, 2000).

3.2 HABITAT AND DISTRIBUTION

The species originates from southeastern Madagascar (Gurib-Fakim, 2006), but has established itself in tropical and subtropical regions of the world. In South Africa it is a common sight in home gardens and disturbed areas such as roadsides.

3.3 USES

3.3.1 Plant parts

Current literature supports the use of all parts of this species, with distinct preferences regarding the plant part or combination of plant parts used. In the Caribbean it is mentioned that plant decoctions are used (Halberstein, 2000), creating the impression that they use the entire plant rather than a specific part. In Mozambique there is a distinct selection of a single part; i.e. roots (Bruschi et al., 2011). Multiple, specified plant parts are used in the Congo and in South Africa. In

the Congo stem and root decoctions are used (Nayak and Pinto Pereira, 2006), and in Maputaland, South Africa, leaves and roots are utilized (De Wet et al., 2008).

3.3.2 Therapeutic and prophylactic application of *Catharanthus roseus*

The medicinal application of this species demands a dual approach. With other species, more often than not, their traditional use overshadows their use in modern medicine. However, *C. roseus* is as well-known as a traditional remedy as it is for its use in modern therapeutics.

Several studies reported the use of this species in the treatment of STIs (Hutchings et al., 1996; Bruschi et al., 2011) and diarrhoea; with roots as a common denominator in these extracts. Ross (2003) reported only the use of roots, whereas roots and stems are used in the Congo (Nayak and Pinto Pereira, 2006) and roots and leaves are utilized in Maputaland, South Africa (De Wet et al., 2008). Bio-active compounds from *C. roseus* are well documented for their anti-bacterial (Sathiya et al., 2008; Ibrahim et al., 2011), anti-fungal (Roy and Chatterjee, 2010) and anti-malarial activity (Wang et al., 2012a). Leaf extracts illustrated good immune-enhancing activity (Patra et al., 2010), and exhibited wound healing properties (Nayak et al., 2007). In Brazil, leaf infusions are used for internal bleeding, scurvy, as a mouthwash for toothache, and the cleansing and healing of wounds (Nayak and Pinto Pereira, 2006; Shivananda, 2006). The possibility exists that flavonoids (Tsuchiya et al., 1996) and triterpenoids (Scortichini and Pia Rossi, 1991) might be involved. These compounds are known for their wound healing properties, mainly due to their astringent and anti-microbial properties; which seem to be responsible for wound contraction and an increased rate of epithelization.

Various plant parts are used to treat chronic diseases, such as DM (Adeyemi et al., 2003; Ross, 2003) and hypertension (Halberstein, 2000; Ross, 2003). The first laboratory investigations into the pharmacological value of *C. roseus*, was based on its traditional use as an oral hypoglaecemic agent (Gurib-Fakim, 2006). Subsequently a number of studies focused on its potential use in DM. In their rat study, Grover et al. (2002), reported a dose-dependent reduction in the blood glucose levels. In addition to this there were no gross behavioral changes or toxic

effects observed up to 4 mg/kg IP. This dose-dependent reduction was also observed when normal and alloxan-induced diabetic rabbits were subjected to leaf juice (Nammi et al., 2003). It is interesting to note that both these rabbit groups showed a significant reduction in blood glucose levels, even if the normal group showed a less pronounced reduction. A plausible explanation of this mechanism involves nuclear receptors. Rau et al. (2006), found that ethanol extracts of *C. roseus* activated the nuclear peroxisome proliferator activated receptors in cultured human cells. These nuclear receptors function as transcription factors and upon their activation they regulate the expression of the genes that ultimately control the lipid and glucose homeostasis as well as adipocyte differentiation. In the treatment of chronic diseases the roots and leaves of this plant can play an important role. These plant parts have confirmed anti-hypertensive (Sottomayor and Ros Barcelós, 2005) and anti-diabetic activity (Nammi et al., 2003).

Interest in this species, from a modern medicine perspective, was sparked by the discovery and subsequent Food and Drug Administration (United States of America) approval of the anticancer drugs vinblastine and vincristine in the early 1960s (Johnson et al., 1963). Since then, very few species have been so intensely investigated. Subsequently it was realized that this species have much more to offer than only its contribution to cancer treatment. The fact that both vincristine and vinblastine are produced from the leaves is supported by various studies investigating its use in chemotherapy (Costa et al., 2008; Ferreres et al., 2010). It is not surprising that with the identification and isolation of various vinca alkaloids, the most prominent therapeutic use of *C. roseus* bio-active constituents involve cancer treatment. Among these alkaloids; Vincristine (Oncovin®, Eli Lilly), Vinblastine (Velbe®, Eli Lilly), and the semi synthetic drugs Vindosin (Eldesine®) and Vinorelbine (Navelbine®, GlaxoSmith Kline) are best known for their anti-cancer properties (Heinrich et al., 2004).They achieve this via inhibition of mitosis by binding to tubulin. Thus there is no spindle formation and a metaphase arrest occurs in the dividing cells (Creasy et al., 1994; Jordan and Wilson, 2004). Tubulin is normally present in one of two forms, either α- or β-tubulin. The vinca alkaloids are very selective for the high affinity site on β-tubulin known as the vinca domain, and binds to it in a rapid and reversible fashion (Gigant et al., 2005). Each of these vinca alkaloids have a

different affinity (Kruczynski et al., 1998) and have different clinical applications, toxicities and effect spectra (Rowinsky and Tolcher, 2001). Vinblastine is useful in the treatment of cancers such as Hodgkin's disease, advanced testicular cancer and breast cancer. Vincristine is also useful in the treatment of Hodgkin's disease, but its primary use includes the treatment of leukemia. Vindosin can also be used in the treatment of leukemia and lung cancer, whilst Vinorelbine is used for ovarian cancer. Vinorelbine is the drug of choice when HIV-positive patients present with Karposi's sarcoma. It works at a lower concentration and has fewer side effects when compared to the alkaloids that are directly derived from the plants (Heinrich et al., 2004).

In general assessing the therapeutic value of any plant species or a specific bio-active constituent in it includes an investigation into its possible mutagenicity and cytotoxicity. Various studies reported on these aspects for *C. roseus* and some of its bio-active constituents. Elgorashi et al. (2003), found that di-chloro-methane (DCM) extracts of *C. roseus* leaves caused frame shift mutations in the *Salmonella typhimurium* strain TA98. These results were confirmed by Verschaeve and Van Staden (2008) who used DCM and methanol extracts from C. roseus leaves, using the same Ames assay. It is well documented that precursor molecules tend to be less cytotoxic towards cancer cells. Sertel et al. (2011) indicated that monomers such as vindoline and catharanthine exhibited weak cytotoxicity, whereas the dimeric vinblastine, vincristine, vindestine and vinorelbine had a high cytotoxicity towards cancer cells. Similar to this, catharoseumine a monoterpenoid indole alkaloid, with its unique peroxy bridge moiety, showed only moderate cytotoxicity against HL-60 cell line (IC_{50} of 6.28 µM) (Wang et al., 2012a).

Other applications include the roots as a source of anti-oxidants, the leaves as an anti-fungal, a serpentine derivative as an acetylcholine esterase (ACE) inhibitor and leaf extracts in wound healing. Pereira et al. (2010a), confirmed that the roots of harvested plants tested positive for the presence of anti-oxidants. Roy and Chatterjee (2010), reported the presence of 5-hydroxy flavone in *C. roseus* leaf extracts. This compound showed antifungal activity when tested against *Fusarium oxysporum*, *Botrytis cinerea* and *Alternaria alternata*. Pereira et al. (2010b) argued that minor structural changes to serpentine may yield a potent and selective ACE

inhibitor; a compound with the potential to change the pharmaceutical management of diseases such as Alzheimer's and myasthenia gravis. Nayak et al. (2007), found significant wound healing when the wounds were treated with leaf extracts. However, there is still some uncertainty as to which constituent/s was responsible for these results.

3.4 PHYTOCHEMICAL CONSTITUENTS

Secondary metabolites produced by plants as a defensive mechanism forms the fundamental basis of the use of herbal remedies. Thus plants, amongst other applications, represent a major therapeutic resource for many humans and animals (Polya, 2003). As such it is important to be cognizant of the various categories that exist regarding these metabolites: alkaloids, phenolics and terpenes. The alkaloid group is widespread in plants and normally names allocated to them ends with –ine. They are basic compounds, generally originating from amino acids (Jaleel et al., 2009), thus the inclusion of a heterocyclic ring containing an N-atom. Plant-derived phenolics designate a very large and diverse group of defensive metabolites, which are recognized by the presence of a hydroxybenzene moiety. These compounds range in complexity from those that have a single ring structure (simple phenolics and quinones) to those with a double ring structure (stilbenes and chalcones), ultimately ending with the very complex triple-ringed structures such as the chromones, anthocyanins, flavonoids and xanthones. The final group, plant terpenes, is appropriately grouped as monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes (Polya, 2003).

In *C. roseus* approximately 150 alkaloids have been isolated and characterized. Probably the best known among these are the indolomonoterpenic alkaloids vinblastine, vincristine (Fig. 3.2 (i)), vinleurosin, vinposidin, and recently added to this group catharoseumine (Wang et al., 2012a). Their prominence relates to their ability to inhibit cell growth and as a consequence of this their value as anti-cancer and/or anti-tumor drugs (Jordan and Wilson, 2004). In both leaves and stems phenolic acids, such as caffeoylquinic acid (Fig. 3.3 (i)), are present in higher amounts than flavonoid derivatives (Pereira et al., 2009). However, leaves had the higher

concentration of the phenolic acid hydroxycinnamic acid. It is of interest to note that when all aerial parts are compared with regard to their phenolic content, that the highest concentration is found in the petals (Pereira et al., 2009). Pereira et al. (2010b) confirmed the absence of hydroxycinnamic acid from root extracts. Coincidently the roots are a very rich source of antioxidants.

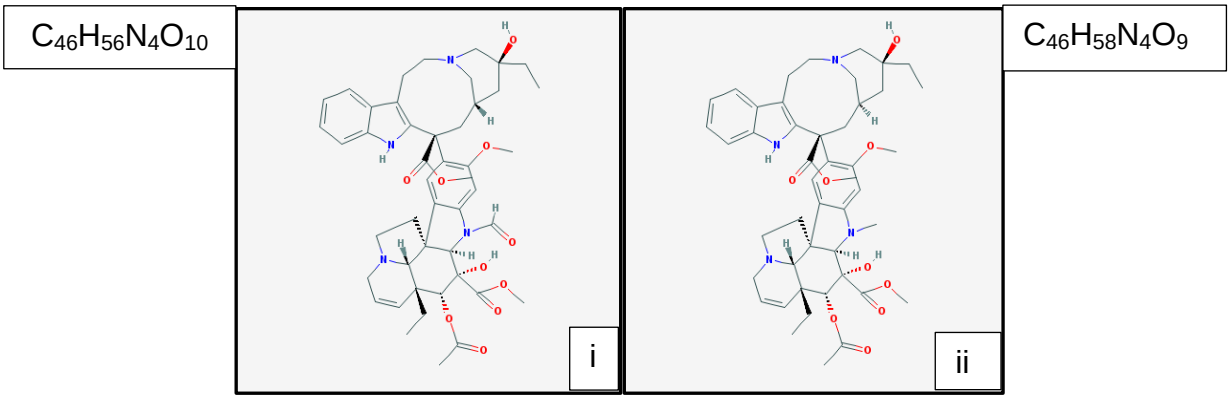

Figure 3.2: Chemical structure of Vincristine (i) and Vinblastine (ii).

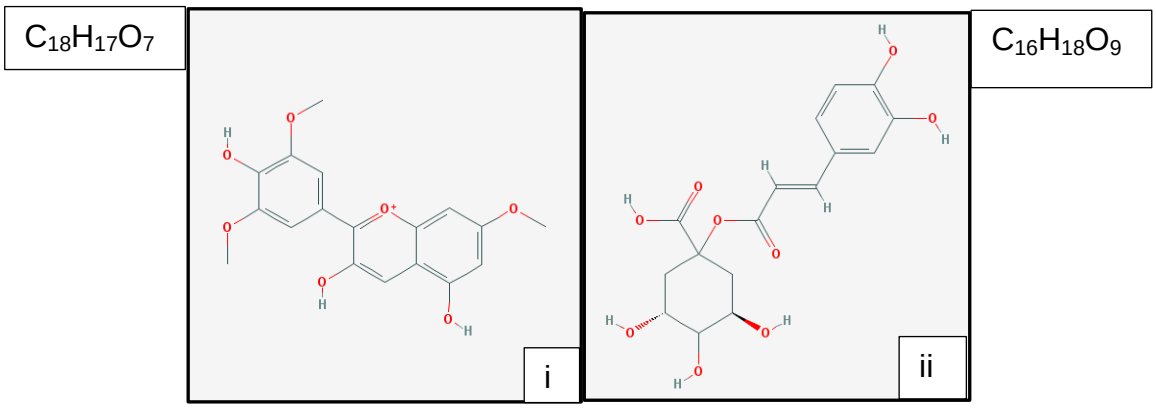

Figure 3.3: Chemical structure of Hirsutidin (i) and Caffeoylquinic acid (ii).

The following eight dimeric indole alkaloids exhibited cytotoxic effects against human breast cancer cell line MDA-MB-23; 14', 15'-didehydrocyclovinblastine, 17-deacetoxycyclovinblastine, 17-deacetoxyvinamidine (Wang et al., 2012b), vinamidine (Boenmann and Kuehne, 1992), leurosine (El-Sayed et al., 1980), catharine (Kutney et al., 1979), cycloleurosine (Honty et al., 1999), and leurosidine (Mukhopadhyay and Cordell, 1981).

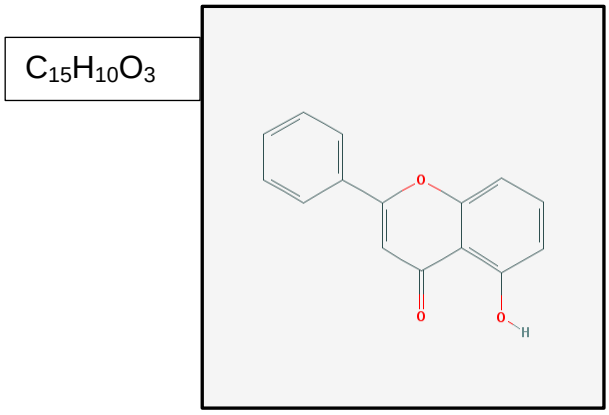

Figure 3.4: Chemical structure of 5-hydroxyflavone.

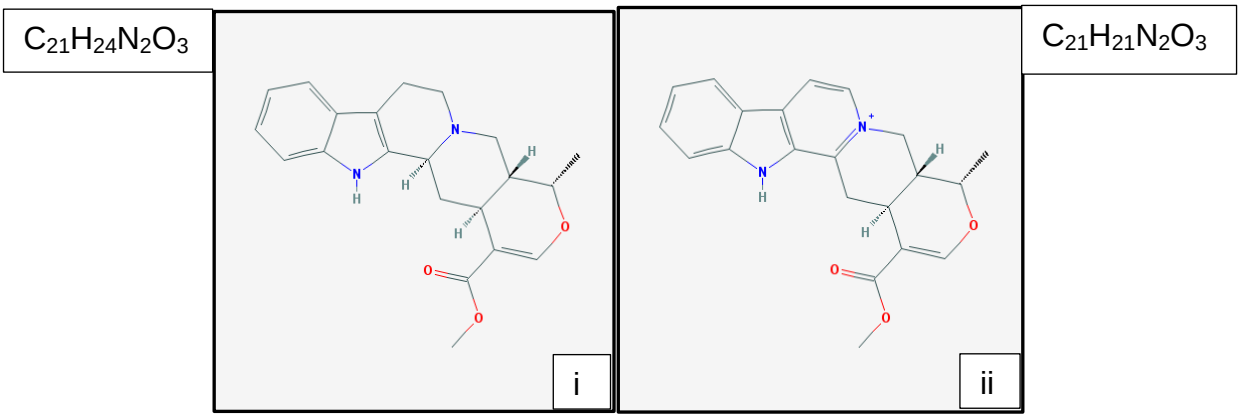

Figure 3.5: Chemical structure of Ajmalicine (i) and Serpentine (ii).

Ajmalicine (Fig. 3.5 (i)), an alkaloid isolated from *C. roseus* roots, is used to treat hypertension and obstructive circulatory diseases (Verpoorte et al., 1991). This effect on the cardiovascular system was recently confirmed in the study by Almagro et al. (2011). They found that it can be used to effectively treat arrhythmias and to improve the cerebral circulation. Similarly, serpentine (Fig. 3.5 (ii)) can be used to effectively treat circulatory diseases, as well as anxiety (Van der Heijden et al., 2004).

Hypoxis hemerocallidea Fisch.Mey. & Ave-Lall.

HYPOXIDACEAE

i

Ga-Mamadila village, Polokwane municipality

ii

Star-shaped flower

iii

Corm and fleshy adventitious roots

iv

Identical triplets?

Figure 4.1: *Hypoxis hemerocallidea*.

Star-flower, African potato (English); Sterblom (Afrikaans); Phela (North Sotho); Lotsane, moli-kharatsa (Sotho)

CHAPTER 4: *HYPOXIS HEMEROCALLIDEA*

4.1 BOTANICAL DESCRIPTION

Hypoxis hemerocallidea (Syn. *H. rooperi*) is a tuberous, geophytic perennial herb, endemic to South Africa. It belongs to the family Hypoxidaceae, which comprises of eight genera and approximately 130 species (Fabian and Germishuizen, 1997).

The *leaves* are long, strap-shaped and slightly hairy (Fig. 4.1 (i)). They are arranged one above the other to form three distinct groups of leaves spreading from the center of the plant.

Two to twelve bright-yellow, star-shaped *flowers* (symmetrical with six petals) are borne on long, slender and unbranched stalks. The flowers are symmetrical with six petals (Fig. 4.1 (ii)). The aerial parts of this species die back during the winter months. The first rains in spring initiate the growth of a new set of leaves and flowering stems from the corm (Nair, 2006).

In South Africa the tuberous rootstock (corm) of this herb is popularly referred to as “African potato” (Van Wyk et al., 2009). These large corms (10–15 cm) (Drewes et al., 2008) vary in colour from dark brown to black on the outside and from light to bright yellow inside (Van Wyk et al., 2009; pers. observ.). It is interesting that when Linnaeus coined the epithet “*Hypoxis* L.” from the Greek words hypo (indicating less than or below) and oxy (sharp), that he focused on the pointed nature of the corm (Linnaeus, 1759 cited by Nair 2006). Corm collection in the present study clearly illustrated that often the corms are not pointed at all, but flat-based to such an extent that the entire plant could be placed upright without any support. The corms have thick, fleshy adventitious roots that arise from the base of the corm (Fig. 4.1 (iii)). According to Nair (2006), the corms can be graded according to size: ≥ 200 g represents one year old seedlings, ≥ 450 g those that are at least three years old, and ≥ 800 g are specimens older than three years.

4.2 HABITAT AND DISTRIBUTION

The species of *Hypoxidaceae* have a geographic distribution in the Southern Hemisphere; predominantly South America, South Africa, Australia and the coastal regions of Asia. They are mostly found growing in meadows, grasslands, low scrubs and mountainous areas (Baker, 1896 as cited by Nair 2006; Greenick, 1968). In southern Africa, *Hypoxis hemerocallidea* is widely distributed in the savanna regions of South Africa, Swaziland, and Zimbabwe (Katerere and Eloff, 2008). It is a predominantly summer rainfall genus and as such a large number of species occur in the eastern region of South Africa (Nair, 2006).

4.3 USES

4.3.1 Plant parts

The tuberous nature of this species creates the impression that only the corms are traditionally used to treat ailments. This observation is not entirely true as existing literature support the use of corms (Drewes et al., 2008; Madikizela et al., 2012) as well as leaves (Aremu et al., 2010; De Wet et al., 2012).

4.3.2 Therapeutic and prophylactic application of *Hypoxis hemerocallidea*

Hypoxis hemerocallidea has been used as an African traditional medicine for a very long period of time. It is argued that if it had been found to be toxic following long term use, it would not have remained a reliable herbal drug frequently used by traditional healers in South Africa (Drewes and Khan, 2004). This line of reasoning seems flawed as a clear definition of what is considered “long term use”, does not really exist in traditional medicine. Most remedies are taken three times per day for a period of a week (Erasmus et al., 2012). There is as yet no sound proof, from the context of traditional medicine that if or when a patient does not consult after completing treatment, that the person was indeed healed. The flip side can either be that the patient did not get better and decided to consult elsewhere or that the person died; not a farfetched concept when it is considered that all plant species,

including *H. hemerocallidea*, contains toxic compounds. However, this being said, this species is currently still used in traditional medicine and the focus of many pharmaceutical studies, not ignoring the various patents already registered (Drewes and Liebenberg, 1987; Allison et al., 1996; Bouic, 1999).

Traditional usage as well as its application in modern medicine, supports the diverse medicinal value of this genus. Traditional usage patterns focus primarily, albeit not exclusively, on issues related to sexual health. The most prominent treatments include HIV/AIDS and related illnesses (Amusan et al., 2007; Corrigan et al., 2011), STIs and urinary tract infections (Buwa and Van Staden, 2006), ulcers caused by ulcerative STIs (De Wet et al., 2012), impotency and barrenness, prostatitis and benign prostatic hyperplasia (BPH) (Drewes et al., 2008). The therapeutic value of the phytosterols, β-sitosterol and stigmasterol, in the management of human health is well established. The combination of β-sitosterol and its glycoside is claimed to be useful in the treatment of BPH. Its mechanism of action can in all probability be ascribed to the inhibition of 5-α-reductase or to the diminished binding of dihydrotestosterone to their receptors in prostatic tissue (Drewes and Khan, 2004). In 5-α-reductase deficient individuals there are an impaired conversion of testosterone to dihydrotestosterone (Kumar and Clark, 1994). According to Rhodes et al. (1993), *H. hemerocallidea* was found to have an IC_{50} of 500 µg/ml on human 5-α-reductase. This supports the use of the species in treating BPH, prostate cancer and male pattern baldness. This enzyme is involved in steroid metabolism, more specifically that of androgens and estrogen; thus any therapeutic agent with the potential to act as a 5-α-reductase inhibitor can be used in the treatment of abnormalities related to this enzyme.

Other traditional applications include the treatment of chronic diseases of life style, such as cardiovascular diseases (Pooley, 1998) and DM (Semenya et al., 2012a); as well as bad dreams, insanity, intestinal parasites (Drewes et al., 2008), lower stomach pain (in a pers. comm. cited by Motha, 2003), and for diarrhoea (Bisi-Johnson et al., 2010).

A number of the traditional uses of *H. hemerocallidea* have been scientifically verified. Bouic and Albrecht (1993) and Bouic et al. (1996) confirmed its use as an immune booster. Zibula and Ojewole (2000) and Ojewole (2006) used various

animal models to confirm the anti-nociceptive (rat and mice), anti-inflammatory (rat hind-paw, fresh egg albumin-induced inflammation) and hypoglaecemic activity (rat) of *Hypoxis* extracts. The anti-nociceptive activity of hypoxoside was evaluated using various tests, such as the tail-flick assay, phenylquinone writhing test and the formalin test, on Swiss-Webster mice. Even though various doses were being used, hypoxoside did not induce any gross behavioral changes. Of interest is that a 20 mg/kg dose, the highest used in this study (Di Giannuario et al., 1993), was capable of reducing the nociceptive response in both the formalin and writhing tests; however, no effect was observed in the tail-flick assay. Two other studies (Zibula and Ojewole, 2000; Ojewole, 2006) assessed, amongst others, the anti-nociceptive activity of *H. hemerocallidea* extracts. These studies did not specifically test the effect of hypoxoside or rooperol; however, their extracts illustrated an increased threshold to pain in both rat and mice models.

In addition to this, Steenkamp et al. (2006) found that ethanolic extracts inhibited COX-1 enzyme activity and aqueous extracts inhibited COX-2 activity, also contributing to the anti-inflammatory repsonse. Ethanolic and aqueous extracts inhibited COX-1 and COX-2, respectively. Laporta et al. (2007) confirmed that rooperol showed COX-1 and -2 inhibitory capacities, which may contribute towards the anti-inflammatory and anti-nociceptive properties of *H. hemerocallidea* extracts. The possibility also exists that due to this anti-inflammatory ability, the traditional use of *H. hemerocallidea* extracts can effectively eliminate an abnormal urethral discharge. Especially if it is considered that the discharge might result from an inflammatory condition rather than from the pathogen itself.

The norlignan glycoside, hypoxoside, is hydrolyzed in the gastrointestinal tract to rooperol (Marini-Bettolo et al., 1982 as cited by Nair, 2006). This conversion is regulated by β-glucosidase, an enzyme produced by intestinal bacteria (Nair, 2006). Animal models, such as mice, confirmed that hypoxoside is not absorbed per se into the circulation since it could not be detected in serum. However, rooperol is absorbed into the circulation. Subsequently, pharmacological studies confirmed that hypoxoside has limited pharmacological activity, whereas rooperol has been shown to be highly active.

Rooperol possesses inhibitory activity on cell growth, and as such is useful as a pro-drug in cancer therapy (Albrecht et al., 1995a). It has also been confirmed that it can induce apoptosis in the HL-60 human promyleucytic leukemia cell line (Albrecht et al., 1995b). However, the molecular basis for this observed cytotoxicity is still not clear. Rooperol also exhibited anti-mutagenic and anti-oxidant activity, as it was found that it actively inhibit mutagenesis (Ames assay), and has the ability to scavenge free radicals ten times more actively than ascorbate ions (Albrecht, 1995).

4.4 PHYTOCHEMICAL CONSTITUENTS

In South Africa the introduction of *H. hemerocallidea* into modern medicine probably started with the entrepreneur R.W. Liebenberg, who in 1967 initiated the use of *Hypoxis* phytosterols (β-sitosterol and its glucoside) to treat BPH (Drewes et al., 2008). The urological preparation Harzol® was originally extracted from *H. hemerocallidea* (Nicoletti et al., 1992). Lowe and Fagelman (1999) reasoned that the presence of β-sitosterol enabled this preparation to enhance the production and secretion of plasminogen activators in isolated epithelial cells. Amusan et al. (2007) phytochemically assessed *H. hemerocallidea* for the presence of tannins, steroids, saponins, polyphenols and glycosides. Their extracts tested negative for flavonoids, anthranoids and alkaloids.

The major phytochemical constituent, isolated from the corms, is the pentenyne derivative named hypoxoside (Fig. 4.2) a norlignan diglucoside (de Smet, 1998), and its active compound rooperol (Drewes et al., 2008). Nair (2006) highlighted the presence of six major phytochemical compounds in *H. hemerocallidea*. Among these hypoxoside was listed as the most known compound. Other listed phytochemicals included daucosterol (Fig. 4.3 (i)), β-sitosterol (Fig. 4.3 (ii)), zeatin (Fig. 4.4), hexosans and pentosans

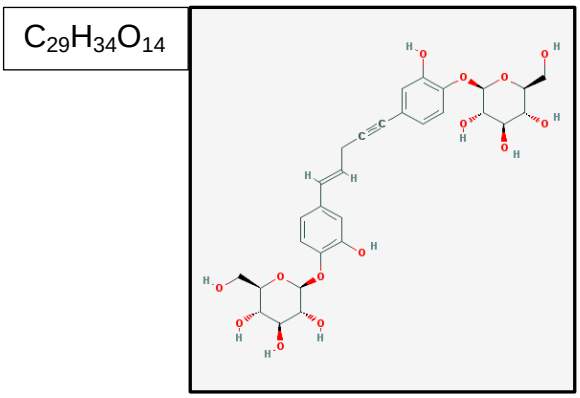

Figure 4.2: Chemical structure of Hypoxoside.

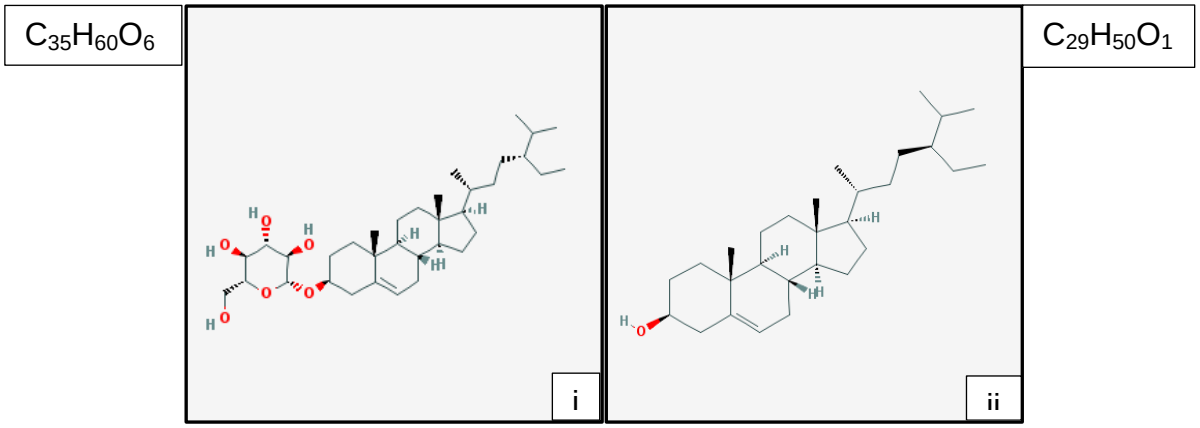

Figure 4.3: Chemical structure of Daucosterol (i) and β-Sitosterol (ii) (Syn. gamma-sitosterol).

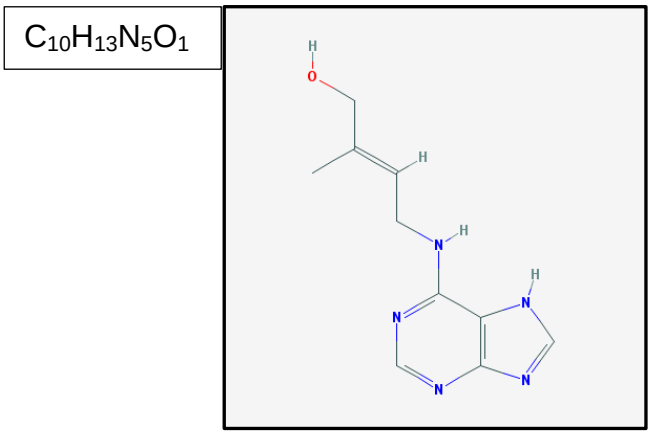

Figure 4.4: Chemical structure of Zeatin.

Daucosterol is a β-sitosterol glycoside. The phytosterols (β-sitosterol) are structurally similar to cholesterol, and their esters reduce cholesterol absorption and reduces low density lipoprotein (LDL) cholesterol. Zeatin is one of the adenine derivatives that are plant growth regulator cytokinins possessing mitogenic and anti-senescent activity in plants (Polya, 2003).

Tribulus terrestris L.

ZYGOPHYLLACEAE

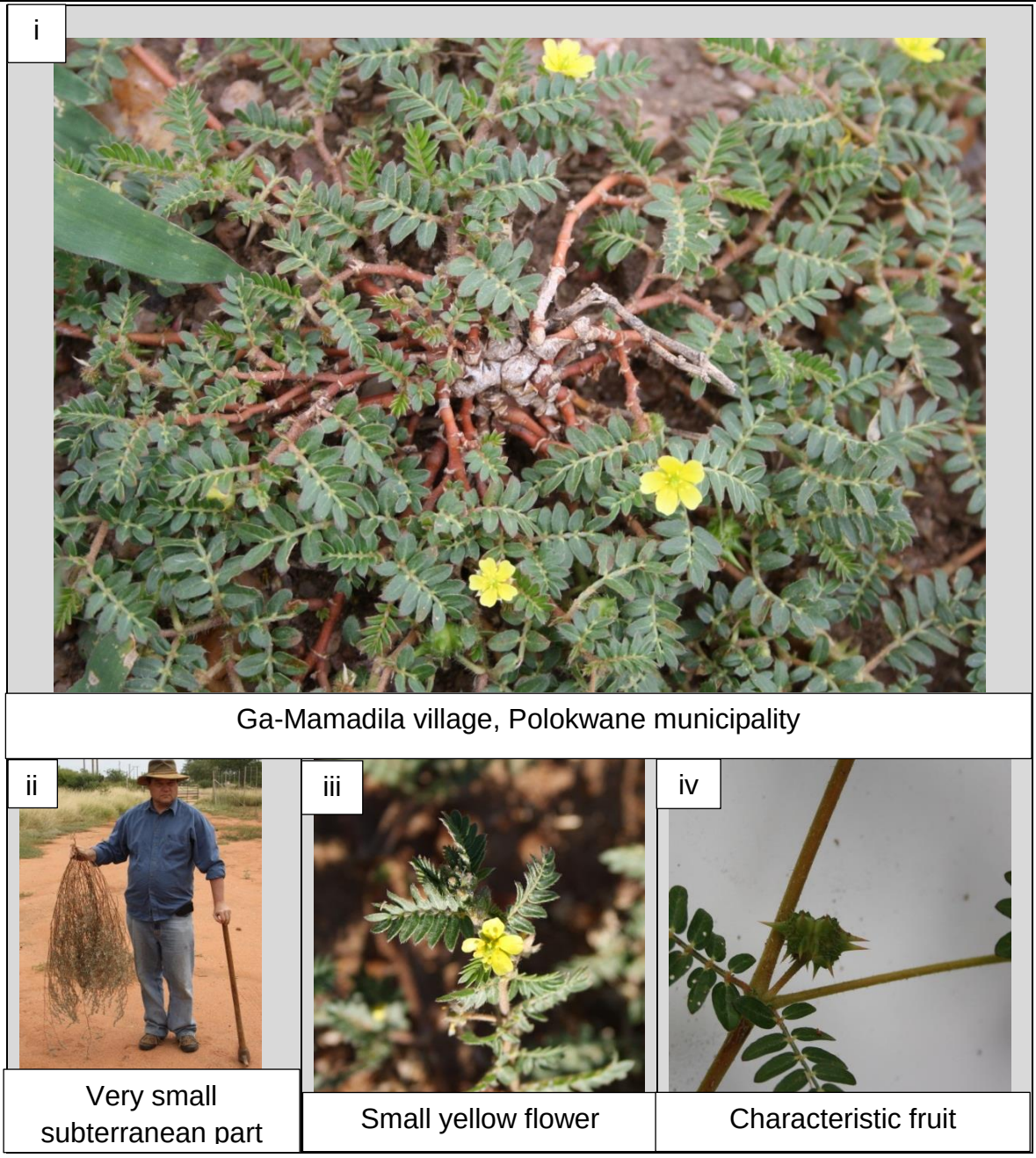

Figure 5.1: *Tribulus terrestris*.

Devil's thorn (English); Duwweltjie, dubbeltjie (Afrikaans), Tshehlo / sehlabammisisi (North Sotho).

CHAPTER 5: *TRIBULUS TERRESTRIS*

5.1 BOTANICAL DESCRIPTION

A widely distributed (Sharifi et al., 2003) herbaceous, perennial species. It is usually prostrate, forming flat patches; with stems radiating from its crown, to a width of between 0 and100 cm (Akram et al., 2011).

Leaves are pinnately compound (Phillips et al., 2006) with leaflets less than 6 mm long (Akram et al., 2011).

Flowers of this species are 4–10 mm wide, with five lemon-yellow petals (Akram et al., 2011).

Numerous stellate-shaped carpel *fruits* are normally present (Phillips et al., 2006). Macrosopic characteristics of the fruits describe them as yellowish-globose and spiny with each having five woody, spiny cocci. Each coccus has four pointed rigid spines; the two larger spines are directed towards the apex and the two smaller ones downward (Akram et al., 2011).

5.2 HABITAT AND DISTRIBUTION

In South Africa *Tribulus terrestris* is an exceptionally common weed, with a very wide distribution (Bromilow, 2001); especially in disturbed areas (pers. obs.).

5.3 USES

5.3.1 Plant parts

Current documented traditional practices include the use of fruits/seeds and sometimes the entire plant. The entire plant is used for both human and animal health purposes. Van der Merwe et al. (2001) highlighted its ethnoveterinary value in the treatment of retained placenta and bloatedness. Georgiev et al. (1988) indicated that the entire plant or the fruit can be used for spermatorrhoea. The seeds/fruits are the most widely used in remedies to treat a magnitude of ailments. Not surprisingly

its greatest utilization and value is found in the oriental medicine. Here fruits/seeds are used to treat ailments such as hypertension and cardiovascular diseases (Lu et al., 1994), eye trouble, edema, abdominal distension, sexual dysfunction (Akram et al., 2011), erectile dysfunction and renal failure (Li, 1983; Capoor, 1990). Adimoelja (2000) also indicated the use of fruits in the treatment of impotence. The seeds are recommended in the treatment of hemorrhage, kidney stones and gout (Shinwari and Khan, 2000). Roots and fruits are reported as useful in the treatment of ailments such as rheumatism, menorrhagia and impotence (Akram et al., 2011).

5.3.2 Therapeutic and prophylactic application of *Tribulus terrestris*

True to human nature, the most prominent ailments covered in the literature were related to optimal functioning of the reproductive system; especially that of the male reproductive system. This was followed by disorders of the cardiovascular and renal systems. Table 5.1 summarizes some of the medicinal applications of *T. terrestris*.

The use of *T. terrestris* extracts to treat decreased free testosterone serum levels is well documented (Brown et al., 2001; Hussain et al., 2009). Its efficacy in the treatment of ED can be attributed to the conversion of the steroidal saponin protodioscin into the weak androgen dehydroepiandrosterone (DHEA) (Adimoelja et al., 2000). The process of steroidgenesis involves the Δ^4 and Δ^5 pathways. In the Δ^5 pathway DHEA is converted into androstenedione which can either be converted to estrone and then to estradiol-17β, or the androstenedione can reversibly be converted to testosterone (Jones and Lopez, 2006). In addition, Neychev and Mitev (2005), highlighted the fact that *T. terrestris* is a natural luteinizing hormone (LH) stimulant, as it stimulates LH release from the pituitary gland. This can also increase testosterone levels, as LH can stimulate the testicular Leydig cells to release testosterone (Jones and Lopez, 2006). Its value in the treatment of various male sexual disorders was further emphasized by Akram et al. (2011) who reported that not only protodioscin, but also dioscin and diosgenin increases free testosterone levels. This study indicated that *T. terrestris* increased testosterone levels in a way unlike that employed by DHEA and androstenedione. It was proposed that instead of acting as a testosterone precursor it might increase LH production. Yet, another

possibility for its use in ED relates to the increased electrical and nitroglycerine induced relaxation of rabbit corpus cavernosa, which is consistent with pro-erectile function (Adaikan et al., 2000). It is therefore fair to argue, that in the light of increased testosterone levels, there will be a subsequent increase in the activity of the Sertoli cells and that spermatogenesis will also increase. A scenario, that offers treatment possibilities to males suffering from oligospermia.

Tribulus terrestris extracts (Jiji et al., 2009), especially crude saponin fractions such as the drug “Xinnnao Shatong” (Lu et al., 1994), has significant therapeutic value in the treatment of cardiovascular diseases (CVD) and dyslipidemia. It was indicated that the beneficial CVD effects could partially be attributed to its ability to raise endothelium derived release of nitric oxide (NO) from the nitrergic nerve endings (Adaikan et al., 2000), as well as direct smooth muscle relaxation (Arcosoy et al., 1998). Sharifi et al. (2003) reported a significant anti-hypertensive effect when aqueous extracts were used. It was suggested that a possible mechanism of action could be the inhibitory effect on angiotensin converting enzyme activity. Nonetheless, vasodilation in their study appears unlikely to be related to inhibition of this enzyme since Arcosoy et al. (1998) was unable to observe any muscle relaxant effect in the rabbit aorta. Even so, similar concentrations of these extracts did inhibit peristaltic movement in sheep ureter and rabbit jejenum, in a dose-dependent fashion. Another prospect, which was not assessed in this study, is that these extracts could have produced their anti-hypertensive effect via dilation of the resistance vessels, thus reducing total peripheral resistance.

Tribulus terrestris has been tested against various pathogens, to assess its bactericidal potential. Awadh Ali et al. (2001) reported activity against Gram-positive bacteria, but not against Gram-negative strains. Mohana et al. (2008), used the cup diffusion method to screen for *in vitro* antibacterial activity. They tested aqueous extracts against eleven bacterial strains and found low antibacterial activity.

Table 5.1: Summary of the most prominent medicinal uses of *Tribulus terrestris*.

Organ system	Ailments and/or uses	Reported uses
Reproductive	Erectile dysfunction	Adaikan et al. (2000); Adimoelja et al. (2000); Gauthaman and Ganesan (2008)
	Increased libido and increased free serum testosterone	Tomova et al. (1981); Brown et al. (2001)
	Aphrodisiac	Majeed and Mahmood (1988); Capoor (1990)
	Morbid leucorrhea	Akram et al. (2011)
	Emission and premature ejaculation	Akram et al. (2011)
	Menorrhagia	Akram et al. (2011)
	Gynaecomastia	Jameel et al. (2004)
Cardiovascular	Hypertension and anti-hypertensive	Majeed and Mahmood (1988); Arcosoy et al. (1998); Sharifi et al. (2003); Mothana and Lindequist (2005)
	Hypercholesterolemia and anti-hyperlipidemic	Arcosoy et al. (1998); Jiji et al. (2009)
	Coronary heart disease and cardiovascular diseases	Wang et al. (1990); Sharifi et al. (2003)
	Edema	Akram et al. (2011)
	Hemorrhage	Shinwari and Khan (2000)
	Astringent	Majeed and Mahmood (1988)
Renal	Diuretic	Arcosoy et al. (1998); Selvan (2008)
	Kidney stones and lithontriptic	Sangeeta et al. (1994); Shinwari and Khan (2000); Akram et al. (2011)
	Kidney failure	Capoor (1990)
	Urinary anti-infectives	Majeed and Mahmood (1988)
Gastrointestinal	Promote peristalsis	Al-Ali et al. (2003)
	Colic pains	Arcosoy et al. (1998)
	Abdominal distension	Akram et al. (2011)
	General disorders	Chemexcil (1992)
	Stomachic	Majeed and Mahmood (1988)

5.4 PHYTOCHEMICAL CONSTITUENTS

Various terpenes, phenolics and alkaloids have been isolated from this species. In an effort to simplify the complexity of bioactive compounds from *T. terrestris*, the following three categories will be used: (i) alkaloids, (ii) phenolics, and (iii) terpenes.
A small number of alkaloids are documented. Among these are furostanol glycoside (Mahato et al., 1981; Wu and Shi, 1999) and harman and norharman (Wink and Van Wyk, 2008). The latter two being β-carboline alkaloids.

A limited number of phenolics, *for T. terrestris*, have been identified. These include kaempherol, which is one of the most important ginko flavonoids; quercetin a flavanol with anti-oxidant activity; and the well-known lignanamides tribulusamide A and B (Li et al., 1998).

The best studied group of phytochemical compounds belongs to the terpenes. Studies by Mahato et al. (1981), Yan et al. (1996), Wu and Shi (1999) and Conrad et al. (2004) identified the following compounds belonging to the terpenes: sitosterol glucoside and β-sitosterol, protodioscin (Syn. furostanol 1), terrestrosins A–E (furostanol type saponins), desglucolanatigonin and desgalactotigonin (steroidal saponins), gitonin (steroidal saponin), tigogenin (sarsasapogenin), spirosta -3, 5-diene (steroidal sapogenin), stigmasterol (phytosterol), diosgenin, and hecogenin and ruscogenin (steroidal sapogenins).

Ziziphus mucronata Willd. subsp. *mucronata*

RHAMNACEAE

i

Ziziphus mucronata, Baltimore area, Blouberg municipality

ii

Young plant, Ga-Choene

iii

Tightly clustered flowers

iv

Characteristic fruit and thorn-configuration

Figure 6.1: *Ziziphus mucronata* subsp. *mucronata*.

Buffalo-thorn (English); Blinkblaar-wag-'n-bietjie (Afrikaans); Mokgalo (North Sotho, Tswana).

CHAPTER 6: *ZIZIPHUS MUCRONATA*

6.1 BOTANICAL DESCRIPTION

This small to medium-sized deciduous tree, with a *height* ranging from 5–10 m in height, can occasionally be taller than 10 m (Fig. 6.1 (i)). It can either have a single trunk or multi-stemmed. Figure 6.1 (i) illustrate the roundish, rather untidy crown, with some branches retaining to the ground (Van Wyk et al., 2011).

Bark is grey to greyish-brown, and fissured into small rectangular sections.

Flowers are present during the summer. They are small, yellow-green and inconspicuous, tightly clustered above each leaf (Fig. 6.1 (iii)). They often produce copious nectar.

Thorns are very sharp, paired spinescent stipules, and are usually present on the twigs (Fig. 6.1 (iv)). However, they are often absent on mature trees. In the pair, one thorn is hooked sharply downwards, and the other curved slightly upward.

Leaves are ovate to broadly ovate with the following dimensions; 3–8 X 2–5 cm. They are shiny green above, and slightly paler green below, with a triplet of veins arising from the base. The undersurface can be either hairless or covered with short, soft, pale-brown, woolly hairs (Van Wyk et al., 2011). The apex is broadly tapering, often with a hair-like tip. The round to lobed base is remarkably asymmetric, with finely-toothed margins around the upper 50–60%.

Fruits are virtually spherical, shiny russet-red ripe berries has an approximate diameter of 10–15 mm. These berries contain a thin layer of rather dry, meal-like pulp, and they often remain on the trees until the leaves fall over the winter-period.

6.2 HABITAT AND DISTRIBUTION

The species occur in a wide variety of habitats, in woodlands, often in alluvial soils along rivers, and frequently in termite mounds. Its distribution is associated with the presence of underground water. However, Van Wyk et al. (2011) indicated that it is not uncommon to find them in semi-deserts.

6.3 USES

6.3.1 Plant parts

This is a widely used medicinal species, with its roots, bark, leaves and even flowers used. Leaves are the most prominent plant part used for medicinal purposes (Watt and Breyer-Brandwijk, 1962; Kayser and Arndt, 2000; Iwalewa et al., 2007; McGaw et al., 2007; Maroyi, 2011). However, some evidence supports the use of roots (Sparg et al., 2000; Adamu et al., 2005; Iwalewa et al., 2007; Bruschi et al., 2011; Maroyi, 2011). It is well-known that the harvesting of roots is ill-advised as it poses a risk to the survival and/or health of a species (Semenya, 2012).

Similar comments regarding the effects of bark-stripping on the survival/health of a species is applicable. Fortunately the use of bark is far less frequent than that of leaves and roots, thus minimizing this harvesting methods' impact (Watt and Breyer-Brandwijk, Bryant, 1966; 1962; Adamu et al., 2005; Iwalewa et al., 2007; McGaw et al., 2007).

Fruits, due to their seasonal availability are quite limited in their medicinal usage. The only reference to its use is the study by Maroyi (2011). Similar to the availability of fruits, flowers are also seasonally limited; the only record of its use is as an ichthyotoxic agent in poison fishing (Neuwinger, 2004).

6.3.2 Therapeutic and prophylactic application of *Ziziphus mucronata*

There seems to be no fixed pattern as to which plant parts are preferred for the treatment of specific ailments. Various authors reported the use of this species in the treatment of infertility (Kayser and Arndt, 2000; Van der Merwe et al., 2001; Mpiana et al., 2008; Maroyi, 2011), menstrual disorders (Arnold and Gulumian, 1984; Mpiana et al., 2008) and STIs (Adamu et al., 2005; Mpiana et al., 2008; Semenya et al., 2013). It is possible that its use in the alleviation of menstrual disorders, probably more related to menstrual flow than to uterine cramping, can at least in part be explained by the findings of Lindsey et al. (1999). These authors found that ethanolic extracts of *Z. mucronata* increased contractility in strips of uterine smooth muscle; a

mechanism that appears to be acetylcholine and not oxytocin related. Findings regarding its use in the treatment of bacterial and fungal infections are at best controversial, especially if the following comment by Ngemenya et al. (2006) is considered. They stated that even if weak *in vitro* activity has been demonstrated that it does not imply a similarly weak response *in vivo* per se. This argument is supported by the notion that some drugs may be more potent *in vivo* due to metabolic transformation of their compounds into highly active intermediates. Some pathogens involved in sexually transmitted diseases are Gram-negative bacteria; however, very little has been done to assess the activity of extracts from *Z. mucronata* against pathogens such as *Neisseria gonorrhoea* or *Chlamydia trachomatis*. This might be attributed to the fact that insignificant activity against Gram-negative pathogens (*E. coli* and *Pseudomonas aeruginosa*) has been reported (McGaw et al., 2007), and as such further investigations were perceived as redundant. Gundidza (1986) reported that aqueous and methanolic stembark extracts showed antifungal activity against *Candida albicans*.

Gastrointestinal tract ailments treated included diarrhoea and dysentery (Arnold and Gulumian, 1984; Iwalewa et al., 2007), stomach ulcers (Arnold and Gulumian, 1984), constipation (Bruschi et al., 2011), enteric conditions (Kudi and Myint, 1999) and abdominal pain (Bruschi et al., 2011; Maroyi, 2011). Its use to relieve pain is well-established; roots are used to treat muscular pain (Bruschi et al., 2011) and toothache (Doke and Vilakazi, 1972), and leaves to relief general pain (Lindsey et al., 1999).

Afflictions of the integument including symptoms related to inflammation are often treated with *Z. mucronata*. Unspecified skin diseases (Kayser and Arndt, 2000), wounds (Luseba and Van der Merwe, 2006; Mpiana et al., 2008; Maroyi, 2011), sores (Van der Merwe et al., 2001; Iwalewa et al., 2007), boils (Palmer and Pitman, 1972; Iwalewa et al., 2007) and burns (Van der Merwe et al., 2001) are more often than not treated using leaves, and from time to time roots.

The typical symptoms of infection and inflammation, quite often the result of abovementioned afflictions, are mostly treated using the leaves of *Z. mucronata*. These symptoms include fever (Arnold and Gulumian, 1984) and glandular swelling (Palmer and Pitman, 1972). However, according to Green et al. (2010) bark and root

can also be used to treat glandular swelling. The cyclopeptide alkaloids in the genus *Ziziphus* is regarded as the most important group of bioactive compounds responsible for its pharmacological actions. It was therefore proposed that the anti-inflammatory activity of these alkaloids be mediated via the inhibition of prostaglandin synthesis (Goyal et al., 2012).

Furthermore, the use of all plant parts has been documented for their medicinal value in the treatment of respiratory ailments. This species is often used to act as an emetic or expectorant for the treatment of chronic coughs (Iwalewa et al., 2007; Bryant, 1966). However, ethanolic leaf extracts have shown activity against *Mycobacterium smegmatis* and *M. tucerculosis* (Mativandlela et al., 2008).

In addition to this Adewusi and Steenkamp (2011) reported good anti-oxidant activity as well as good ACE inhibitor activity. Even though not completely unexpected, concerns exist regarding reports of cytotoxicity and mutagenicity. Taylor et al. (2003) reported that *Z. mucronata* is highly toxic and causes both DNA damage and chromosomal aberrations. Furthermore, Elgorashi et al. (2003) employing both di-chloromethane and methanol leaf extracts, reported that the methanolic extracts exhibited mutagenic activity when the bacterial Ames assay was used.

6.4 PHYTOCHEMICAL CONSTITUENTS

Phytochemical constituents identified in the genus include flavonoids, tannins, sterols, saponins, pectin, glycosides, alkaloids and triterpenoic acids (Goyal et al., 2013). According to Goyal et al. (2012), the cyclopeptide alkaloids, such as Ziziphine-N (Fig. 6.2) are regarded as the compounds responsible for the observed pharmacological actions of *Ziziphus* spp. More than 170 cyclopeptide alkaloids have been identified, more than 80 of them in the genus *Ziziphus* (Rahman et al., 2001). These peptide alkaloids have macrocyclic 13–15-membered rings incorporating various peptide (-CO-NH-) links (Polya, 2003). Rahman et al. (2001), further indicated that the cyclopeptides identified among the *Ziziphus* spp. included 35 with 13-membered rings, 39 with 14-membered rings, and only seven with 15-membered rings.

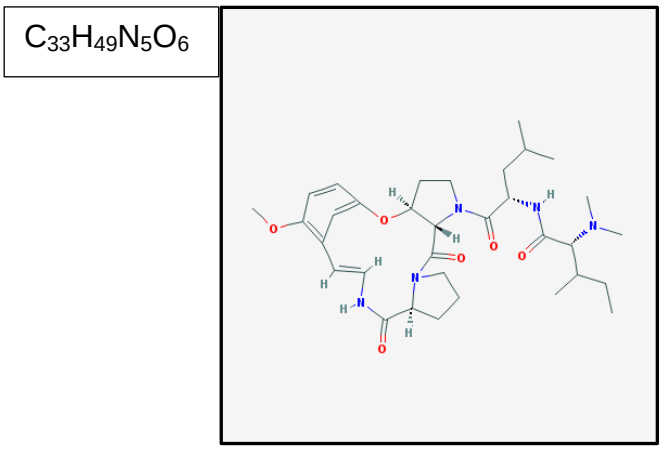

Figure 6.2: Chemical structure of the cyclopeptide Ziziphine-N.

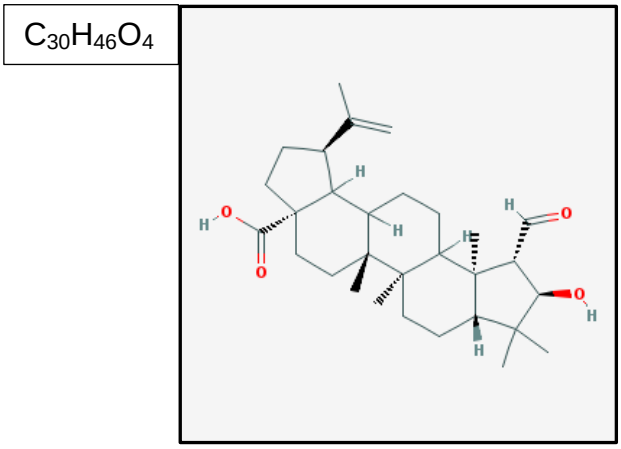

Figure 6.3: Chemical structure of Zizyberanalic acid (II) a pentacyclic triterpenoid from *Ziziphus mucronata.*

The pentacyclic triterpenoid Zizyberanalic acid (Fig. 6.3), and 2,3-dihudroxyl-up-20-en-28-oic acid have been isolated from *Z. mucronata* (Moloto, 2004). Various phenolics, especially anthocyanins (Mpiana et al., 2008), as well as the cyclopeptide mucronine-D (Van Wyk et al., 2009) have been isolated from this species.

7. GENERAL AIM AND OBJECTIVES

7.1 Aim of the study

An initial aim was to determine the phytochemical profile of older and younger *Aloe marlothii* subsp. *marlothii* plants, in an effort to link plant age with potential antimicrobial efficacy. However, the main aim of the current study was to investigate the rationale for the medicinal application of selected plant species against specific STIs; by assessing their *in vitro* anti-microbial activity, as well as the impact of various boiling intervals on phytochemical profiles.

7.2 Objectives of the study

The objectives of the study are to:

a) Conduct a pilot study, assessing phytochemical profiles via thin-layer chromatography (TLC), to confirm whether small or tall *A. marlothii* plants should be used in the anti-microbial assays;

b) Assess the antimicrobial efficacy, of aqueous extracts prepared from *A. marlothii* roots and leaves, and the subterranean parts of *Z.mucronata*, *H. hemerocallidea*, *T. terrestris* and *C. roseus*, using the micro-dilution assay;

c) Determine whether the combination of different plant species, or plant parts, would improve their antimicrobial efficacy;

d) Assess, via HPLC-DAD analysis, the impact of various boiling intervals, as noted among the Bapedi when aqueous extracts are prepared from the plant species used in the current study, on the availability of alkaloids, phenolics and terpenoids;

e) To combine HPLC-DAD data and antimicrobial findings to identify the compound groups most probably involved in the detected activities.

8. GENERAL HYPOTHESES

a) Plant parts from younger *A. marlothii* plants are better sources of phytochemicals than those from more mature plants;

b) Employing different boiling intervals will affect the bio-active profiles of aqueous extracts and establish the existence of time-dependent extraction relationships;

c) Multi-plant / multi-plant part aqueous extracts are more effective antimicrobials than single plant / single plant part aqueous extracts.

9. COMPOSITION OF THIS THESIS

The composition of this thesis entails a functional prologue that includes relevant information regarding the primary bacterial pathogens and plant species that will be the focus of the research (Chapter 1 to 6). It should be noted that when this work refer to *Aloe marlothii* it is with specific reference to subsp. *marlothii*, similarly any reference to *Ziziphus mucronata* will indicate subsp. *mucronata*.

Chapters 7 to 13 are presented in article format as certain journals have been identified as relevant to the publication of the specific contents of chapters. A comprehensive reference list covering all chapters appears at the end of the thesis. This format was decided on as it saves time in preparing manuscripts to be published, and due to the mutli-disciplinary nature of this research it would also credit the various researchers who assisted me in the completion of certain sections.

In essence, excluding the pilot study in chapter 7, there are two major focus areas. Chapters 8 to 10 investigates the antimicrobial efficacy of selected plant species. Chapters 11 to 13, following the same order for the plant species assessed in Chapters 8 to 10, reports on the phytochemical profiles of the various aqueous extracts and how they have been altered during the different boiling times.

The epilogue, chapter 14, is the concluding chapter.

Slight deviations from the normal manuscript format (Chapters 7 to 13) have been implemented. Some colour has been added to the various tables.

It should be noted that the Harvard referencing method is used throughout.

10. SCOPE OF THE STUDY

The scope of the study was defined by a number of aspects. The decision to collect these species fom a single geographical location was to standardize climatological and geological influences. The selection of plant species is based on previous surveys conducted among the Bapedi in the Limpopo Province. *Catharanthus roseus* was included due to its exclusive use in the treatment of gonorhoea; *Aloe marlothii* subsp. *marlothii* due to the fact that some traditional healers used roots, others used leaves and others preferred combining these plant parts. Interest regarding *Ziziphus mucronata* stemmed from the fact that it was often mentioned in combination with species such as *Hypoxis hemerocallidea* and *Tribulus terrestris*.

The choice of microbes was more challenging. *Neisseria gonorrhea* was the primary pathogen as it is well known as a causative agent in the development of an abnormal urethral discharge. *Proteus vulgaris*, a gram-negative bacterium, was included as a comparison for *N. gonorrhea*. *Staphylococcus aureus*, which is gram-positive, was selected not for its impact on the reproductive system but due to the fact that it would enable us to assess the impact of our extracts on both gram-negative and gram-positive bacteria. The only fungus included, and known for its impact on the reproductive system was *Candida albicans*.

The selection of appropriate and relevant methods to address the focus of this work was based on simplicity. The aim was to assess traditional preparation of decoctions, hence the focus on water as the only extraction medium and the specific boilng time intervals as preferred by the Bapedi. High performance liquid chromatography was employed to determine the alterations in phytochemical profiles and the micro-dilution method to evaluate antimicrobial efficacy of the crude extracts.

The primary focus of this study was to assess the impact of different boiling periods on the phytochemical profile of crude extracts, and how this affected the antimicrobial efficacy of these extracts. High performance liquid chromatography and micro-dilution enabled us to obtain relevant and appropriate results. Therefore, the inclusion of mass spectrometry was deemed unnecessary.

11. LIMITATIONS

This study had a number of limitations that defined its scope. Extending the sampling of plants to a number of geographical areas would have complicated this study. This approach would have induced a number of variables related to climate and soil composition.

The antimicrobial assessment of crude extracts against *Chlamydia trachomatis* was not included in the present study. Co-infections involving *C. trachomatis* and *N. gonorrhea* are well-documented. In the rural setting the identification of STI pathogens is not possible; therefore, it is plausible that both of these organisms can be implicated in the pathogenesis of such an infection (i.e., abnormal urethral discharge). Thus this study could not definitively rule out the use of low activity crude extracts in the treatment of STIs.

SECTION 1
ANTIMICROBIAL ASSESSMENT

Traditional ADJ. of, relating to, or in accord with *tradition*: thus referring to the handing down of reports, principles, legends, customs, information, etc.

Aloe marlothii subsp. *marlothii*: A pilot study to assess the possible therapeutic potency of leaves from young and old plants in the Nobody area, Limpopo Province, South Africa

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ABSTRACT

Aloe marlothii (Asphodelaceae) is a widely distributed southern African medicinal plant. Ethnobotanically, this species is extensively used by Bapedi traditional healers in the Limpopo Province of South Africa. However, limited phytochemical information is available for this species. This study therefore investigated six plants (3 taller than 2 m and 3 shorter than 2 m), collected in close vicinity to one another, for possible therapeutic differences, via phytochemical screening and analysis, as well as the option of using dead leaves as a substitute for fresh leaves. In this study preliminary biochemical analysis of phytochemicals was combined with phytochemical analysis using Thin Layer Chromatography. Findings indicated that both young (≤1 m) and older plants (≥ 2 m) had the potential to be used in the treatment of various ailments. However, the use of younger plants was found to be the more preferred option, as they constantly yielded larger numbers of compounds. The possibility of using dead leaves as substitutes for fresh leaves was not overwhelmingly supported by the results. Yet, it is believed that these leaves could be used to great effect in the treatment of, amongst others, cardiovascular diseases.

Key words: *Aloe marlothii* subsp. *marlothii*, Bapedi, Phytochemical analysis, Secondary metabolites, Thin layer chromatography.

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7.1 INTRODUCTION

The majestic *Aloe marlothii*, a winter-flowering species (May to September), is widespread and conspicuous where it grows in warm valleys and on hill slopes (Cousins and Witkowski, 2012). In southern Africa this species can easily be distinguished from all other single-stemmed aloes by the horizontal racemes with erect flowers (Van Wyk and Smith, 2008). There is an inclination to grow on north-facing rocky hills; however, they are often noted in the bush, where similarly to the rocky hills, they frequently form extremely dense, impenetrable stands (Bredenkamp and Van Vuuren, 1987). They can reach heights of up to 6 m (Van Wyk and Smith, 2008), have a single stem and are protected from fire by persistent skirts of withered leaves around the stem (Bond, 1983). The bearded appearance is created by the presence of dead, dry leaves covering a significant section of the stem, especially in younger plants.

In southern Africa the traditional use of various *Aloe* species as therapeutics is well-described. For example, in Zimbabwe *Aloe globuligemma* Pole Evans (Kambizi and Afolayan, 2001) and *Aloe greatheadii* Schönland (Maroyi, 2011) are used to treat sexually transmitted infections (STIs) and constipation. In Mozambique *Aloe parvibracteata* Schönland is also used in the treatment of STIs (Bruschi et al., 2011). Among the Swati in Swaziland, *Aloe arborescens* Mill. leaves are used to treat diabetes mellitus; while leaves of *A. marlothii* subsp. *marlothii* as well as *Aloe saponaria* (Aiton) Haw. are used to treat ailments related to the cardiovascular system (Amusan et al., 2007). The Zulu ethnic group in South Africa use *A. marlothii* subsp. *marlothii* leaves in the treatment of respiratory infections (York et al., 2011). They also use leaf pulp applied to the breasts of breastfeeding mothers to hasten weaning; and a mixture prepared form roots and leaves to treat internal parasites such as roundworm (Watt and Breyer-Brandwijk,1962).

In the Limpopo Province (South Africa) *A. marlothii* subsp. *marlothii* forms an important part of the traditional health care system. The VhaVenda ethnic group use this species' leaves to treat blood in the faeces (Mabogo, 1990), while Bapedi traditional healers in the Capricorn District apply leaves extensively to treat STIs

such as gonorrhoea and HIV (Erasmus et al., 2012), as well as diabetes mellitus (Semenya et al., 2012).

Even though this species is frequently mentioned as a medicinal resource, very little is known regarding its phytochemical profile. High Performance Liquid Chromatography (HPLC) indicated the presence of both aloeresin A and aloesin in leaf exudate (Van der Bank et al., 1995). Thin layer liquid chromatography (TLC), using methanol as an extractant, resulted in the isolation of a chromone 7-O-methylaloeresin A as well as an anthrone 5-hydroxyaloin A 6'-O-acetate (Bisrat et al., 2000). An investigation into the phenolic content of *A. marlothii* subsp. *marlothii* roots resulted in the isolation of a number of anthraquinones and pre-anthraquinones, such as chrysophanol, asphodelin, aloesaponarin I, and aloesaponol I and II (Van Wyk et al., 1995).

In the quest to record and protect indigenous knowledge, it is unfortunate that traditional medical practice is shrouded in secrecy, as most traditional healers are reluctant to disclose the art of their practice (Amusan et al., 2007). In line with this, it is not clear whether Bapedi traditional healers use leaves from young or more mature plants. It is known that they do harvest fresh leaves and then allow a recovery period for the specific plant (Semenya, 2012). The medicinal use of dry dead leaves around the stem has not been documented among the Bapedi. However, the VhaVenda (pers. comm.) do use the dead leaves to treat ailments, such as STIs. This highlights the potential therapeutic value of these dead leaves, that when harvested is less destructive than collecting green leaves. The purpose of this chapter is therefore to compare the phytochemical profile of leaves collected from young plants with that of more mature plants, and to investigate the possibility of using dry dead leaves from the skirts as a substitute for green leaves.

7.2 MATERIALS AND METHODS

7.2.1 Plant collection

Aloe marlothii subsp. *marlothii* leaves (n = 2 per plant) were collected in January 2013, from the Nobody area (S 23°53'52.0"; E 29°37'08.0") within the Capricorn

District of the Limpopo Province, South Africa. All six plants (3 with a stem length ≥ 2 m, and 3 with a stem length ≤ 1 m) were collected within the same week, thus minimising the impact of seasonality. A conscientious effort was made to collect leaves from a young and more mature plant growing within a distance of 2 m from each other. This was done to reduce the possible contribution of soil variations towards fluctuations in bio-active profiles. All plants were also collected within a 50 x 50 m quadrat. For consistency, fresh and dead leaves were collected from the western side; which was indicated as the most preferred side for leaf collection (Semenya et al., 2012). Dead leaves refer to the brown, dry leaves that were collected from the stem. Collected leaf material were cut into smaller pieces, dried and ground into fine powder (1 mm mesh) using a hammer mill (Mikro-Feinmühle-Culatti, Jamke & Kunkel IKA-Labortechnik, Germany).

7.2.2 Extraction procedure

Bio-active compounds were extracted using 1.0 g of finely ground plant material dissolved in 10 ml of n-hexane, dichloromethane (DCM), acetone, and methanol (according to solvent polarities ranging from non-polar to more polar) in polyester centrifuge tubes. Tubes were vigorously shaken for 10 minutes in a series 25 incubator shaker (New Brunswick Scientific Co., Inc) at 100 rpm, thereafter the extracts were filtered into pre-weighed labelled bottles. The process was repeated in triplicate to exhaustively extract the compounds, thereafter the extracts were combined. The solvent was removed under a stream of cold air at room temperature.

7.2.3 Phytochemical analysis

The extracted chemical constituents of the extracts were analyzed by separation using aluminium-backed TLC plates (Fluka, silica gel F_{25}). The TLC plates were developed in saturated chambers using mobile phases of different polarities, namely; benzene/ethanol/ammonia hydroxide [BEA] (non-polar/basic), chloroform/ethyl acetate/formic acid [CEF] (intermediate polarity/acidic), and ethyl acetate/methanol/water (40:5.4:4) [EMW] (polar/neutral), in line with the method of

Kotze and Eloff (2002). The chromatograms were viewed under ultraviolet light (254 and 365 nm) for fluorescing compounds, and later sprayed with vanillin-sulphuric acid reagent (0.1 g vanillin (Sigma®): 28 methanol: 1 ml sulphuric acid) and heated to 110^{o}C for optimal colour development.

7.2.4 Preliminary biochemical analysis of phytochemicals

The leaves were examined for the presence of the following components; and appropriate colour changes were used for interpretation.

7.2.4.1 Alkaloids

The Drangendoff's reagent method described by Harborne (1973) was used. Powdered leaves (0.2 g) were extracted with 95% ethanol in a Soxhlet extractor for six hours. The ethanolic extracts were evaporated to dryness using a vacuum evaporator at 45°C. The residue was re-dissolved in 5 ml of 1% HCl and 5 drops of Drangendoff's reagent (solution of bismuth sub-nitrate and potassium iodide) was added. The samples were observed for the formation of a coloured (yellowish) precipitate to draw inference.

7.2.4.2 Saponin

The persistent frothing test for saponin of Odebiyi and Sofowora (1978) was used. One gram of powdered plant material was mixed with 30 ml tap water. The mixture was vigorously shaken and heated. Samples were observed for the formation of a persistent froth to indicate the presence of saponins.

7.2.4.3 Phlobatannin

Powdered leaf samples (0.2 g) were dissolved in 10 ml of distilled water and filtered. The filtrate was boiled with a 2% HCL solution. The samples were observed for the formation of a coloured precipitate to draw inference.

7.2.4.4 Tannins

The method of Trease and Evans (1989) was adopted. Powdered leaf samples (0.5 g) were dissolved in 5 ml of distilled water, then boiled gently and cooled. The solution (1 ml) was transferred into a test tube and 3 drops of a ferric chloride solution was added and observed for brownidh green or a blue-black colouration.

7.2.4.5 Terpenes/ terpenoids

The Salkowski test was used to test for the presence of terpenes. Powdered leaf samples (0.5 g) were mixed in 2 ml chloroform, followed by cautiously adding 3 ml concentrated sulphuric acid (H_2SO_4), to form a layer. A reddish brown colouration of the inter face was considered indicative of a positive result.

7.2.4.6 Steroids

Acetic anhydride (2 ml) was added to 0.5 g powdered leaf of each sample, followed by the addition of 2 ml of sulphuric acid. Colour changes were observed to draw inference.

7.2.4.7 Cardiac glycosides

The Keller-Killiani test was used, where 0.5 g of plant extract was added to 5 ml of water. The mixture of 2 ml of glacial acetic acid, containing one drop of 0.1 % ferric chloride solution, was added to the diluted extract. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicates the presence of a deoxysugar characteristic of cardenolides (Borokini and Omotayo, 2012).

7.2.4.8 Flavonoids

Diluted ammonia (5 ml) solution was added to a portion of the aqueous filtrate of each plant extract, followed by the addition of concentrated sulphuric acid. Colour changes were observed to draw inference.

7.3 RESULTS

Table 7.1 presents the distribution of the various secondary metabolites in the leaves of the plants selected for this study, while Tables 7.2 to 7.4 summarises the TLC results. Table 7.5 shortly summarises the number of bands as depicted in Tables 7.2 to 7.4.

7.4 DISCUSSION

Ethnobotanical studies, due to historically incomplete records, often create more questions than answers. This is at present the scenario with the harvesting of *A. marlothii* subsp. *marlothii* leaves by Bapedi traditional healers in the Limpopo Province of South Africa. From previous studies (Erasmus et al., 2012; Semenya, 2012) it was difficult to establish which size of plant was targeted by Bapedi for leaf harvesting; especially where STI treatment is concerned. It was therefore important, as a preliminary screening for a prelude to a more comprehensive study, to determine whether a phytochemical rationale exist for utilising younger plants rather than older plants; or *vice versa*.

At first glance results from our study seemed to reflect the findings of Bisrat et al. (2000). Their study on the same species, but from a different location (Vivo) in the Limpopo Province, found that the intra-species mosaic pattern of variation in bio-active profiles, in a single population, can at best be described as erratic. Furthermore, this level of variation seemed to be independent of geographical distribution. This observation, by Bisrat et al. (2000), contradicts the report by Ye et al. (2005) who concluded that the content of active ingredients is determined by environmental and ecological conditions. Nonetheless, regarding this disparity, in the

current single population study all young and older plants were collected in close proximity of each other; in an effort to minimize the impact of environmental and ecological factors.

CHAPTER 7
Aloe marlothii A.Berger subsp. *marlothii* pilot study

Table 7.1: Summary of the phytochemical screening of *Aloe marlothii* leaves collected from young and more mature plants in the Nobody area, Capricorn District, Limpopo Province.

Chemical constituents	Young plants (stem length ≤ 1.0 m)						Mature plants (stem lengths ≥ 2.0 m)					
	Fresh leaves			Dead leaves			Fresh leaves			Dead leaves		
	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
Cardiac glycosides	+	+	+	+	+	+	+	+	-	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	-
Phlobatannins	-	-	-	-	-	-	-	-	-	+	-	+
Saponins	-	-	+	-	-	-	-	-	+	-	+	-
Steroids	+	+	+	+	+	-	+	+	+	+	+	+
Tannins	-	-	-	+	+	+	+	-	-	+	+	+
Terpenes	+	+	-	+	-	+	-	+	-	-	+	+

Key: + = present; - = absent. P = plant, followed by its numerical value.

Table 7.2: Summary of the observed TLC banding patterns for young and old *Aloe marlothii* subsp. *marlothii* plants; using BEA as the solvent system.

YOUNG PLANTS																												
Rf	PLANT 1								PLANT 2								PLANT 3								Total	Grand Total
	Hex		DCM		Ace		Meth		Hex		DCM		Ace		Meth		Hex		DCM		Ace		Meth			
	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D		
0.88	X	X				X				X		X		X			X								7	
0.75		X															X								2	
0.64		X															X		X		X				4	
0.57													X		X										2	
0.52	X		X		X												X		X		X				6	
0.48	X	X	X		X		X			X		X	X	X	X		X		X		X		X		14	
0.42	X		X	X	X	X	X	X	X		X		X		X		X	X	X	X	X	X	X		18	
0.37		X		X		X		X		X		X		X		X		X					X		10	**112**
0.33	X		X		X					X	X	X	X	X	X	X									10	
0.26	X		X		X												X	X							5	
0.20									X								X		X		X				4	
0.17			X		X				X		X		X		X		X								7	
0.13	X						X		X		X		X		X		X	X	X		X				10	
0.08	X		X		X		X		X		X		X		X										8	
0.03			X		X												X		X		X				5	
112		5		2		3		2		4		4		4		2		4		1		1		0	Total Dry	

	8		8		8		4		5		5		7		7		11		7		7		3		Total Fresh	
	12:28								14:24								6:28								Grand Total	
OLD PLANTS																										
0.88																		X				X			2	
0.64																		X							1	
0.60																	X		X		X		X		4	
0.52	X		X		X		X						X				X		X		X		X		9	
0.48									X	X	X	X	X	X	X	X		X		X		X			11	
0.42	X	X	X	X	X	X	X	X		X		X		X		X	X	X	X	X	X	X	X	X	20	
0.37																			X		X		X		3	
0.33						X		X	X	X		X		X		X									7	**87**
0.26									X	X		X	X	X		X									6	
0.20													X			X		X		X	X	X			6	
0.17					X				X	X							X		X		X		X		7	
0.13			X		X		X		X	X	X		X		X										8	
0.08														X											1	
0.03			X		X																				2	
		1		1		2		2		6		4		5		4		4		2		4		1	Total Dry	
87	2		4		5		3		5		2		5		2		5		6		6		6		Total Fresh	
	6:14								19:14								11:23								Grand Total	

Key: Hex = n-Hexane; DCM = Di-chloro-methane; Ace = Acetone; and Meth = Methanol. Grand totals are expressed as ratios (Total Dry:Total Fresh), depicting the number of bands observed in each case.

Table 7.3: Summary of the observed TLC banding patterns for young and old *Aloe marlothii* plants; using EMW as the solvent system.

YOUNG PLANTS																												
Rf	PLANT 1								PLANT 2								PLANT 3								Total	Grand Total
	Hex		DCM		Ace		Meth		Hex		DCM		Ace		Meth		Hex		DCM		Ace		Meth			
	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D		
0.90	X																								1	
0.82	X	X		X		X		X																	5	
0.75	X		X		X		X						X												5	
0.69			X		X												X		X		X		X		6	
0.63	X			X		X		X				X	X	X	X										8	
0.59																	X		X	X	X	X	X	X	7	
0.54	X				X																				2	
0.52					X								X	X	X		X				X				6	
0.48					X	X	X						X		X					X		X			7	**98**
0.44														X		X						X			3	
0.40	X		X	X	X	X	X															X			7	
0.35						X							X	X	X		X		X		X		X		8	
0.31			X		X		X							X											4	
0.26				X		X						X	X	X	X	X	X		X	X	X	X	X	X	14	
0.20			X										X		X										3	
0.17						X								X		X					X	X		X	6	
0.06						X							X	X	X						X		X		6	

98		1		4		8		2		0		2		8		3		0		3		6		3	Total Dry	
	6		5		7		4		0		0		8		7		5		4		7		5		Total Fresh	
	15:22								13:15								12:21								Grand Total	
OLD PLANTS																										
0.82																		X		X		X		X	4	
0.75			X		X				X		X		X		X										6	
0.63			X		X		X					X		X		X				X		X		X	9	
0.59													X		X						X		X		4	
0.54				X	X	X		X	X											X		X		X	8	
0.52												X		X		X									3	
0.48					X																				1	
0.44						X						X		X		X									4	
0.40					X		X		X		X														4	**72**
0.35											X		X	X	X							X			5	
0.31					X																	X			2	
0.26												X	X	X	X	X				X	X	X		X	9	
0.20					X	X																			2	
0.17									X					X	X	X				X		X			6	
0.11																				X		X	X		3	
0.06																				X		X			2	
		0		1		3		1		0		4		6		5		1		7		9		4	Total Dry	
72	0		2		7		2		4		3		4		5		0		0		2		2		Total Fresh	
	5:11								15:16								21:4								Grand Total	

Table 7.4: Summary of the observed TLC banding patterns for young and old *Aloe marlothii* subsp. *marlothii* plants; using CEF as the solvent system.

YOUNG PLANTS																												
Rf	PLANT 1								PLANT 2								PLANT 3								Total	Grand Total
	Hex		DCM		Ace		Meth		Hex		DCM		Ace		Meth		Hex		DCM		Ace		Meth			
	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D		
0.80	X																X	X							3	73
0.73	X		X	X	X	X			X				X												7	
0.67	X								X	X	X	X	X	X	X	X	X		X		X				12	
0.62																	X								1	
0.52	X		X		X		X										X		X		X		X		8	
0.49				X		X		X			X		X		X										6	
0.45	X		X		X		X				X		X		X		X		X		X				10	
0.41																				X		X		X	3	
0.38												X		X		X									3	
0.18													X		X										2	
0.14						X							X	X	X										4	
0.06	X		X	X	X	X	X					X	X	X	X	X				X		X		X	14	
73		0		3		4		1		1		3		4		3		1		2		2		2	Total Dry	
	6		4		4		3		2		3		7		6		5		3		3		1		Total Fresh	
	8:17								11:18								7:12								Grand Total	
OLD PLANTS																												

0.80			X		X		X		X																4	78
0.73			X		X		X		X			X		X		X	X		X		X		X		11	
0.67			X		X		X		X	X							X								6	
0.62						X																			1	
0.55				X	X	X		X																	4	
0.52			X		X		X																		3	
0.49		X		X		X		X	X		X		X		X										8	
0.45			X		X		X												X		X		X		6	
0.41									X		X	X	X	X	X	X									7	
0.38																		X	X	X	X	X			5	
0.28				X	X	X		X						X											5	
0.23																					X		X		2	
0.18												X		X		X									3	
0.14					X									X											2	
0.06					X		X		X		X	X	X	X	X	X					X		X		11	
78		1		3		4		3		1		4		6		4		0		3		4		2	Total Dry			
	0		5		9		6		6		3		3		3		2		2		2		2		Total Fresh			
	11:20								15:15								9:8								Grand Total			

Table 7.5: Summary of the sum totals of the various bands detected during TLC analysis.

	PLANT 1								PLANT 2								PLANT 3								Total
	Hex		DCM		Ace		Meth		Hex		DCM		Ace		Meth		Hex		DCM		Ace		Meth		
	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	F	D	
Young /BEA	8	5	8	2	8	3	4	2	5	4	5	4	7	4	7	2	11	4	7	1	7	1	3	0	32 80
Old/ BEA	2	1	4	1	5	2	3	2	5	6	2	4	5	5	2	4	5	4	6	2	6	4	6	1	36 51
Young /CEF	6	0	4	3	4	4	3	1	2	1	3	3	7	4	6	3	5	1	3	2	3	2	1	2	26 47
Old/ CEF	0	1	5	3	9	4	6	3	6	1	3	4	3	6	3	4	2	0	2	3	2	4	2	2	35 43
Young /EMW	6	1	5	4	7	8	4	2	0	0	0	2	8	8	7	3	5	0	4	3	7	6	5	3	40 58
Old/ EMW	0	0	2	1	7	3	2	1	4	0	3	4	4	6	5	5	0	1	0	7	2	9	2	4	41 31
Total	22	8	28	14	40	24	22	11	22	12	16	21	34	33	30	21	28	10	22	18	27	26	19	12	

Key: F = freshly harvested leaves; D = brown, dry, dead leaves collected from the stem area.

CHAPTER 7

Aloe marlothii subsp. marlothii pilot study

In this study the presence of various secondary metabolites was confirmed via appropriate tests. The formation of a greenish-black colouration was considered a positive test for tannins. In this study, tannins were almost exclusively found in the dry, dead leaves collected from young and more mature plants; very seldom in the fresh leaves. This observation was unexpected as Borokini and Omotayo (2012) in their assessment of 23 Nigerian plant species reported the presence of tannins in all their freshly collected leaf samples; however, *A. marlothii* was not included in their test panel. Tannins are complex polycyclic phenolics that exhibit, amongst others, antibacterial (Borokini and Omotayo, 2012) and anti-inflammatory (Okwu and Okwu, 2004) capabilities. It should therefore be useful in the treatment of certain bacterial STIs.

Saponins, especially steroid saponins, are glycosides of spirostane triterpenoid sapogenins that are generally non-toxic and have a foaming and detergent propensity (Polya, 2003). Therefore, the use of the persistent frothing test to indicate either their presence or absence. The distribution of saponins in the current study was at best inconsistent. However, its presence in the fresh leaves of both the young and more mature plants (P3); as well as its absence in the younger and more mature plants (P1 and P2) is of interest, as it supports the possibility that strata composition might be a contributing factor. This is a plausible explanation as young and older plants with similar designations (P1, P2 and P3) were collected in close proximity of each other.

The presence of phlobatannins is confirmed by the formation of a red precipitate. Only the dead leaves of some of the older plants tested positive for this secondary metabolite. Its astringent and styptic properties (Borokini and Omotayo, 2012) will contribute to an enhanced blood clotting response.

Positive results for terpenes or terpenoids, include the formation of a reddish-brown colouration of the interface. Terpenoids are composed of isoprenyl (C_5) units, and can be classified as monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}) that includes steroids, cardiac glycosides and saponins, and tetraterpenes (C_{40}) (Polya, 2003; Wink and Van Wyk, 2008). From a physiological and therapeutic point of view this group of substances are quite diverse in their

effects. In phytomedicine they are often used as a result of their antibacterial, antifungal and anti-inflammatory capabilities (Wink and Van Wyk, 2008). Even though no fixed pattern for the presence of terpenes existed, current findings indicated its presence in fresh and dead leaves of young and more mature plants. It is well-known that increased numbers of *C. albicans* can result in vaginitis; symptomatically identified by an abnormal vaginal discharge (Jones and Lopez, 2006). Terpenes are noted for their antifungal as well as anti-inflammatory activities (Polya, 2003), therefore has the ability to alleviate related symptoms.

The presence of flavonoids, in eleven of the tested samples, was confirmed via the formation of a yellow precipitate. Plant phenolics, especially flavonoids, exhibit amongst others antimicrobial (Cushnie and Lamb, 2005), anti-inflammatory and antiseptic activity (Serafini et al., 2010). This is of tremendous therapeutic value for ethnic groups, such as the Bapedi, who utilises plant material high in flavonoid content. A previous report (Erasmus et al., 2012) on Bapedi phytomedicine speculated on the improvement of symptoms (abnormal urethral discharge) related to certain STIs. It was reasoned that this improvement could have been related to either the antibacterial or anti-inflammatory activity of the plant material used; or a combination of both. It would therefore seem credible to argue that the reported antimicrobial, and inhibitory effect of phenolics on both COX and 5-LOX (Polya, 2003), would indeed endorse the use of *A. marlothii* subsp. *marlothii* in the treatment of certain STIs.

Eleven of the twelve samples tested positive for cardiac glycosides. This was achieved using the Keller Killiani test, where the presence of a brown interface, accompanied by a violet ring below it and a greenish ring at the lowest part was considered positive. As the name implies, this group of substances exhibit primarily cardiovascular activities; mainly because they have the ability to inhibit Na^+, K^+-ATPase (Wink and Van Wyk, 2008). Cardiac glycosides are best known for their positive inotropic activity; which is of great value in the treatment of heart failure. The inhibition of this ionic pump results in an increased intracellular Ca^{2+} concentration that promotes stronger cardiac muscle contraction. Not only will it affect cardiovascular activities; the inhibition of Na^+, K^+-ATPase will also influence the

optimal functioning of the nerve cells, therefore impacting of neurotransmission. With these capabilities it is not surprising that extracts containing these compounds have been widely used as poisons (Philippe and Angenot, 2005).

This is, to the best of our knowledge, the first study of this nature on a single population of *A. marlothii* subsp. *marlothii*; assessing the phytochemical profile of the entire leaf. This is in contrast to previous reports on this species, where a leaf exudate was used (Bisrat et al., 2000), and where the anthraquinone and pre-anthraquinone levels in roots (Van Wyk et al., 1995) were determined. From the leaf exudate 5 compounds were identified; 7-0-Methylaloeresin A, 5-Hydroxyaloin A 6'-O-acetate, 7-O-methylaloesin, 5-Hydroxyaloin A and aloesin. Van der Bank et al. (1995), using HPLC reported the presence of aloesin and aloeresin A in a leaf exudate. The chemotaxonomic study by Van Wyk et al. (1995) identified five major compounds in the roots of *A. marlothii* subsp. *marlothii*; chrysophanol, asphodelin, aloesaponarin I and aloesapanol I and II. The first two was reported to be characteristic constituents of the subterranean metabolism of *Aloe*.

It is well-documented that traditional healers prefer water as an extraction medium (Runyoro et al., 2006; York et al., 2011; De Wet et al., 2012). Because water is polar the extraction procedure will have an effect on the isolation of non-polar compounds, and thus the contribution of such extracts towards the treatment of certain ailments. This argument is supported by Masoko and Eloff (2006) who highlighted the fact that the success of compound isolation is largely dependent on the solvent used. It is therefore reasonable, even if water was not used as a solvent in this study, to reflect on two other solvents with similar polarities; methanol which is strongly polar in nature and acetone an intermediate towards polar solvent. In combination with the polar mobile phase, EMW, both of these solvents performed well in isolating compounds from fresh and dry dead leaves collected from young plants. When using these solvents in combination with EMW to isolate compounds from older plants, observations were dubious at best.

Thin layer liquid chromatography clearly indicated that acetone almost always extracted the highest number of compounds. Observations furthermore suggest that this trend was not influenced by either the age of the plant, or whether fresh or dry

dead leaves were used. However, that being said, it is relevant to note that using various solvents and mobile phases more compounds were extracted from fresh leaves than from the dry, dead leaves. This is suggestive of a loss of some bioactive compounds during the drying period, as it is well known that variations exist in the type and amount of phytochemicals present in fresh and dried leaves (Sofowora, 2008). These findings without doubt indicate that future studies, focussing on the medicinal relevance of leaf extracts from this species, should strongly consider the use of leaves from younger plants. Such studies should also focus on the use of the dry, dead leaves as a substitute for fresh leaves. These leaves contain, amongst others, high levels of phenolics and can, when used, contribute to the sustainable utilisation of this species.

The absence of bands were limited, and mostly confined to the combination of EMW with either hexane or DCM. This type of response was not unexpected as the combination of a polar mobile phase with a non-polar solvent could fail to extract constituents. What was unexpected is the fact that this trend was not consistently observed with other combinations; the only exclusion being the combination of BEA with methanol (P3).

7.5 CONCLUSIONS

It can be surmised that both young and older plants contain a large diversity of chemical compounds; enough to justify their ethnomedicinal use. However, further studies focussing on quantitative analysis of the phytochemical constituents present in these extracts is of paramount importance; especially when assessing the value of this species in the treatment of bacterial STIs, where microbial resistance is currently of great concern. Future studies should preferably focus on using younger plants; as they yielded far larger numbers of compounds than their older counterparts. Even though TLC analysis does not entirely support the use of dry, dead leaves as a substitute for fresh leaves, they seem to contain compounds that might proof invaluable in the treatment of some diseases or related symptoms.

CHAPTER 8

Antimicrobial activity of *Aloe marlothii* subsp*. marlothii* crude aqueous extracts

Aloe marlothii subsp. *marlothii*: *In vitro* antimicrobial efficacy versus boiling intervals of leaves and roots

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ABSTRACT

Aloe marlothii is widely used by the Bapedi in the Limpopo Province, South Africa, to treat sexually transmitted infections. However, scientific validation regarding the antimicrobial efficacy of this species, based on traditional preparation protocols, is lacking. The current study was designed to investigate the *in vitro* antimicrobial activity of aqueous extracts. Aqueous extracts were prepared according to the boiling protocols favoured by the Bapedi. Antimicrobial activity was determined using the minimum inhibitory concentration (MIC) assay against the following STI-related pathogens: *Candida albicans* (ATCC 10231), *Neisseria gonorrhoea* (ATCC 49226) and *Proteus vulgaris* (ATCC 6380). Aqueous extracts of *Aloe marlothii* leaves and roots exhibited good antibacterial and antifungal activity. MIC values ranged from 0.09 to 6.25 mg/ml for *N. gonorrhoea*, 0.39 to 12.5 mg/ml for *P. vulgaris* and 0.39 to 0.78 mg/ml for *C. albicans*. Root extracts were more effective than those prepared from leaves. However, combining roots with leaves exhibited the best antimicrobial activity. Antimicrobial activity observed in aqueous extracts prepared from the leaves and/or roots of *Aloe marlothii* validates its use in traditional medicine. Further pharmacological assessment is required to identify the active principles.

Key word: *Aloe marlothii*, Bapedi, Boiling, *Candida albicans*, *Neisseria gonorrhoea*, *Proteus vulgaris*.

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CHAPTER 8

Antimicrobial activity of *Aloe marlothii* subsp*. marlothii* crude aqueous extracts

8.1 INTRODUCTION

Natural products with therapeutic properties have been used for as long as human civilization exists; and fossils date this usage back to roughly 60 000 years ago (Fabricant and Farnsworth, 2001). Traditional medicine as a modality that focuses predominantly on the use of natural products has become tremendously popular and is widely approved. In this regard the World Health Organisation (WHO, 2011a) estimated that approximately 70–95% of the world's population in developing countries relies solely on plants for their health care requirements.

The succulent genus *Aloe* (Family: Asphodelaceae) is endemic to Africa, and is well-known for its medicinal application. In southern Africa, *Aloe marlothii* a large single-stemmed, winter-flowering plant forms a conspicuous element of the landscape. It is widespread in grassland and savannah biomes (Van Wyk and Smith, 2008), where it often occurs in extremely dense, impenetrable stands (Bisrat et al., 2000). This species has a distinct preference for rocky, north facing slopes and mountainous areas (Bredenkamp and Van Vuuren, 1987). It has been used in traditional medicine for the treatment of ailments such as; intestinal parasites (Watt and Breyer-Brandwijk, 1962), gastrointestinal disorders (Roberts, 1990), cardiac problems (Amusan et al., 2007), sexually transmitted infections (De Wet et al., 2012), respiratory infections (York et al., 2011) and diabetes mellitus (Semenya et al., 2012). Traditional treatment regimens involving herbal extracts, as a therapeutic resource, are less expensive, more readily available, and their utilization is based on extensive indigenous knowledge and expertise (Street et al., 2008).

Documentation of indigenous knowledge through ethnobotanical studies is of great importance as it not only contributes to sustainable utilisation and conservation of plant resources (Muthu et al., 2006); it also contributes towards a better understanding of the traditional medicine practice. It is quite common, in the selection of a suitable species, for further pharmacological assessment, to carefully observe the use of natural resources in folk medicine. Information pertaining species usage by a specific ethnic group is extremely important (Rates, 2001); as it will

reflect on various aspects such as preparation and extraction methods, as well as dosage and administration. Despite relatively well-documented ethnobotanical literature, scientific validation of extract preparation procedures, efficacy and phytochemistry has only recently emerged, and such information regarding South African medicinal plants is currently limited (Van Vuuren, 2008).

A significant proportion of the existing scientific literature focus on the bactericidal (De Villiers et al., 2010; Green et al., 2010; Mulaudzi et al., 2011) and fungicidal activity (Shai et al., 2009; Amoo et al., 2011; Mahlo et al., 2013) of South African medicinal plants. Even though the genus *Aloe* is well presented in the literature; *Aloe marlothii* has received limited attention regarding its ethnobotany (York et al., 2011; De Wet et al., 2012; Semenya et al., 2012); phytochemistry (Van Wyk et al., 1995; Bisrat et al., 2000) and antimicrobial potential (McGaw et al., 2000; Luseba et al., 2007; Naidoo et al., 2013).

Bacterial and fungal resistance have increased dramatically over recent decades (Graybill, 1996; Wright, 2010); mostly as a result of widespread and incorrect usage of antibiotics and antifungals. Subsequently, an urgent need arose for the development of novel antibacterial (Gibbons, 2005) and antifungal (Masoko et al., 2007) agents to address the current failure of therapeutics in the treatment of infectious diseases. The first step towards achieving this goal is the *in vitro* evaluation of plant extracts for antimicrobial and antifungal properties. Previously published ethnobotanical studies (Erasmus et al., 2012; Semenya, 2012), highlighting the disparity in preferred boiling time among Bapedi traditional healers, prompted this study. Therefore, this chapter will focus on the impact of boiling time, as noted among the Bapedi, on the bactericidal and fungicidal potential of aqueous extracts of *A. marlothii* subsp. *marlothii*.

8.2 MATERIALS AND METHODS

8.2.1 Sample collection

Plant materials were collected in February 2013, from Ga-Mamadila village, Capricorn District, Limpopo Province (S 23°47'17.8"; E 29°13'36.4"). Voucher specimens were collected and deposited at the Larry Leach Herbarium (UNIN) of the University of Limpopo. Duplicate leaf and root material was collected, during summer, from three plants growing in close proximity of each other; thus in the same soil strata.

8.2.2 Extraction

8.2.2.1 Preparation of plant extracts

In this study only fresh plant material was used. After collection the leaves and roots were cut into smaller pieces. Aqueous extracts of *A. marlothii* subsp. *marlothii,* was collected after specific boiling periods, and were subsequently screened for antimicrobial activity against three bacterial strains; two Gram negative (*Neisseria gonorrhoea* ATCC 49226 and *Proteus vulgaris* ATCC 6380) and one Gram-positive (*Staphylococcus aureus*). Their fungicidal activity was also assessed via the inclusion of *Candida albicans* (ATCC 10231). These pathogens, with the exception of *Staphylococcus aureus* (ATCC 25923), were specifically selected due to their ability to affect the epithelial lining and mucous membranes of the urogenital tract. However, it should be recognised that *S. aureus*, even though not strictly speaking a causative agent in STI etiology, causes co-infection with STIs (Buwa and van Staden, 2006); therefore its inclusion in the current test panel.

Preparing the leaf extracts; 425 g of material was added to 2.7 L of boiling tap water. Similarly, 426 g of root material was boiled for the preparation of the extracts. During the preparation of the multi-extract 232 g of leaf material was added to 235 g roots, which was boiled together. At specific time intervals (T_{00}, T_{05}, T_{10}, T_{15} and T_{20}

minutes) 500 ml of boiling liquid was removed, and filtered through Whatmann No. 1 filter paper. No extra water was added, thus no topping-up, to ensure maximum yield of bioactive compounds. Filtered extracts were frozen; where after the water was removed via freeze drying (VIRTIS, United Scientific (PTY) Ltd.). The dried, concentrated extracts were used to prepare 50 mg/ml stock solutions which were used in the micro-dilution assays.

Time 0 (T_{00}) was considered the removal of a 500 ml volume within 15–20 seconds after adding the plant material to the boiling water. The other samples were collected at 5 minute intervals succeeding T_{00}.

8.2.3 Antimicrobial activity

8.2.3.1 Bacterial and fungal cultures

The choice of pathogens for investigation was based on STIs commonly associated with an abnormal urethral discharge. These were *N. gonorrhoeae* and *Proteus vulgaris* two Gram-negative bacteria, and *Candida albicans* a Gram-positive fungus.

Neisseria gonorrhoeae was obtained from ATCC as KWIK-STIK® plus microorganism. The bacteria were inoculated onto chocolate agar (Oxoid GC agar base), which was supplemented with 2% (W/V) of haemoglobin and 1% (v/v) of Vitox supplement; where after it was incubated for almost 48 h at 37 ˚C in 5% CO_2. A number of colonies of pure culture were collected from an overnight culture and suspended in 5 ml Mueller-Hinton (MH) broth. The turbidity of this cell suspension, at 540 nm, was adjusted by adding either MH broth or organism as required, until the turbidity of the suspension was equivalent to that of a 0.5 McFarland $BaSO_4$ standard, with an approximate inoculum size of $1x10^6$ CFU/ml.

Candida albicans, *P. vulgaris* and *S. aureus* were inoculated onto nutrient broth. With the exception of *C. albicans* (48 h), the incubation period was 24 h. The turbidity of all suspensions, at 540 nm, was adjusted by either adding nutrient broth or cultured media until the turbidity of the suspension was equivalent to a 0.5 McFarland standard.

8.2.3.2 Microdilution method

The minimum inhibitory concentration (MIC) values were determined using the micro-titre plate dilution method (Eloff, 1998). In general all assays were performed in triplicate; the only exception being some of the T_{00} samples, where the yield of active compounds was so small that only duplicate assessments could be performed. Stock solutions of the plant extracts were made up to 50 mg/ml using distilled water; dimethyl sulfoxide (DMSO) was not required as the concentrates were initially prepared from aqueous solutions. Nutrient broth (100 µl) was pipetted into all wells of the micro-titre plate. Thereafter, stock solutions (100 µl) of each of the prepared extracts were transferred into the first row (row A) of the micro-titre plate. Serial dilutions were performed, starting from 12.5 mg/ml to 0.097 mg/ml. A standard volume of 100 µl of culture was added to all 96 wells, and the micro-titre plates were then incubated at 37 °C for the appropriate period of time, as required by the specific pathogen. Following the period of incubation, 40 µl of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) (Sigma Chemical Company, St. Louis, MO) was added to all wells. After addition of INT the micro-titre plates were incubated for 30 minutes at 37 °C. The INT viability indicator was added to determine visually where microbial growth changed the colour of the solution; where a red-pink colour indicated viable bacteria and clear-yellow indicated inhibition. The inclusion of negative controls ensured that the relevant nutrient or MH broth were not contaminated. To confirm antimicrobial susceptibility, Ciprofloxacin was used as a positive control at a starting stock concentration of 0.25 mg/100 µl.

8.3 RESULTS

Table 8.1 presents the impact of boiling time on the bactericidal and antifungal activity of various crude extracts prepared from *A. marlothii* subsp. *marlothii*. The MIC values at the various boiling intervals are expressed in mg/ml.

CHAPTER 8

Antimicrobial activity of *Aloe marlothii* subsp*. marlothii* crude aqueous extracts

Table 8.1: The effect of boiling time on the bactericidal and antifungal activity (MIC = mg/ml) of crude extracts from different parts of A*loe marlothii*.

	Leaves					Roots					Leaves and roots				
Time (minutes)	T_{00}	T_{05}	T_{10}	T_{15}	T_{20}	T_{00}	T_{05}	T_{10}	T_{15}	T_{20}	T_{00}	T_{05}	T_{10}	T_{15}	T_{20}
N. gonorrhoea	1.56	1.56	6.25	6.25	6.25	3.12	3.12	1.56	**0.78**	**0.78**	**<0.09**	**0.78**	**0.78**	**0.78**	**0.78**
P. vulgaris	**0.78**	1.56	**0.78**	1.56	1.56	**0.39**	**0.39**	**0.39**	**0.78**	**0.78**	**0.39**	**0.39**	**0.78**	12.5	12.5
C. albicans	**0.39**	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**	**0.39**	**0.39**	**0.78**	**0.78**	**0.78**	**0.78**	**0.78**
S. aureus	1.56	3.12	3.12	12.5	12.5	1.56	1.56	**0.78**	**0.78**	**0.78**	**0.19**	**0.78**	**0.78**	**0.78**	**0.39**
Ciprofloxacin	<0.09 mg/ml against all tested pathogens														

8.4 DISCUSSION

Comparing the findings of this study with other relevant studies (Ndhlala et al., 2009; Mulaudzi et al., 2011; Naidoo et al., 2013), is challenging as all previous studies used dried plant parts as opposed to fresh plant material, and their aqueous preparations were aimed at laboratory procedures rather than traditional preparation protocols.

8.4.1 Antibacterial activity

The antibacterial MIC values of the various extracts all showed varying degrees of activity. Good bactericidal activity of a crude extract, as indicated by Fabry et al. (1998), is reflected in a MIC of ≤8 mg/ml. However, in this study; similar to that of Muluadzi et al. (2011), a MIC of ≤ 1 mg/ml were considered to be indicative of excellent antibacterial activity. Our MIC values ranged from 0.09 to 6.25 mg/ml for *N. gonorrhoea*, 0.39 to 12.5 mg/ml for *P. vulgaris*, and 0.19 to 12.5 mg/ml for *S. aureus*.

In general Gram-positive bacteria normally exhibited a higher level of susceptibility, than Gram-negative bacteria, to plant extracts. This difference in response is often attributed to the structural dissimilarities of the cell walls of Gram-negative bacteria when compared to that of Gram-positive bacteria (Piddock, 2006).

However, in the current study this trend was not consistently observed as susceptibility varied not only between Gram-negative and Gram-positive bacteria, but the two Gram-negative bacteria responded differently to the aqueous extracts. It is evident that the explanation to these observed responses are complex in nature and it is suggested that not only the membrane composition but also the specific phytochemical profile of the extracts are important factors to be considered in further assessments.

The predominantly preferred use of *Aloe* leaves in the treatment of diseases with a bacterial origin is well-documented; especially in the treatment of STIs. In Zimbabwe the leaves of *A. greatheadii* Schönland is used to treat gonorrhoea (Maroyi, 2011); in Mozambique *A. parvibracteata* Schönland leaves to treat venereal

diseases (Bruschi et al., 2011) and in South Africa *A. marlothii* subsp. *marlothii* leaves are used to treat STIs (De Wet et al., 2012). Our investigation into the Bapedi custom of variable boiling times, using *A. marlothii* subsp. *marlothii* leaves clearly illustrates that these aqueous extracts are not very effective against *N. gonorrhoea* or *S. aureus*; they also exhibited only partial noteworthy activity against *P. vulgaris.* However, these extracts were highly effective against *C. albicans*. If it is considered that the traditional healers use the disappearance of the abnormal, smelly urethral discharge as an indicator of the efficacy of their remedies, then the following are plausible explanations; that the infections are mostly caused by *C. albicans*, or that the alleviation of symptoms are based on an effect not related to either the antibacterial or antifungal efficacy of the extracts. Inflammation of epithelial linings or mucous membranes can result in the presence of a purulent discharge; thus if an extract possesses anti-inflammatory potential, it can relieve symptoms related to such a physiological response.

The antimicrobial assessment of root extracts undeniably illustrated their superiority when compared to the leaf extracts. Noteworthy inhibition, throughout the boiling protocol, was observed for both *P. vulgaris* and *C. albicans*. Of interest is that all three bacterial strains reached a MIC of 0.78 mg/ml at T_{15} and this was sustained up to the end of the boiling protocol (T_{20}). Furthermore where *N. gonorrhoea* and *S. aureus* started with relatively insignificant MIC values both concluded at the same level. In contrast to this the MIC range for *P. vulgaris* started at 0.39 mg/ml and concluded at 0.78 mg/ml, similar to that of the other two bacterial strains, yet a decrease in inhibitory levels within its own range. Boiling evidently changed the composition of the aqueous extracts to such an extent that *P. vulgaris* became less susceptible to it. Analysis of these extracts via HPLC will assist in understanding how the bio-active profile changed in relation to boiling time; and how this contributed to the increased efficacy observed against *N. gonorrhoea* and *S. aureus* and the decreased efficacy against *P. vulgaris*.

The use of a combined extract, adding both roots and leaves to the water, dramatically altered the antibacterial activity. Almost all the extracts, excluding *P. vulgaris* T_{15} and T_{20}, exhibited noteworthy activity. The synergistic interaction

observed for *N. gonorrhoea* ($T_{00–15}$) is relevant; especially the fact that T_{00} (0.09 mg/ml) had the lowest MIC detected for pathogens tested in this study. Its relevance relates to the fact that the individual components could not elicit this level of activity, whereas its combination far exceeded any expected response. Extended boiling decreased the efficacy of the extracts towards *P. vulgaris*; with indications of antagonism present. The possibility should be considered that extended boiling of the combined extract could have destroyed the active principle/s involved in earlier responses ($T_{00–10}$), or that extended boiling released other compounds that suppressed the antimicrobial activity of these active principles (antagonism). The most pronounced synergistic effect was observed for *S. aureus*. Where the addition of leaves did not alter the MIC value (T_{10} and T_{15}, 0.78 mg/ml) observed for either roots or the combined extracts, the combination of roots with leaves did add value to the antibacterial activity observed at $T_{00, 05, 20}$. The dynamics of this enhancement of antibacterial activity are not clear and needs further investigation to determine its phytochemical origin.

8.4.2 Antifungal activity

Antifungal activity of aqueous extracts, prepared from leaves and roots of *A. marlothii* subsp. *marlothii*, against *C. albicans* in general demonstrated noteworthy fungicidal activity. The MIC of the extracts ranged from 0.39 to 0.78 mg/ml. The very low MIC observed at T_{00} (leaves) supports the possibility that extended boiling (≥ 5 minutes) of fresh leaves is not a prerequisite for antifungal activity. When compared to the root extracts this level of activity (0.39 mg/ml) was only apparent after boiling for 15 minutes; an effect that lasted up to the completion of the test protocol. It is reasonable to argue that this phenomenon might result from a specific compound found in both of these plant parts, but requires extended boiling to become accessible from root material. However, it is more likely that completely different compounds are at work; especially when the studies by Bisrat et al. (2000) and Van Wyk et al. (1995) are considered. Van Wyk et al. (1995) clearly illustrated that some compounds, such as asphodeline, are characteristic of subterranean metabolism in

Aloe. Bisrat et al. (2000) reported tremendous inter and intra-species variation with regard to the presence of chromones and anthrones in leaf extracts. Unexpectedly, the combination of roots and leaves did not result in a synergistic effect, irrespective of the boiling time. It was anticipated that the lower MIC observed at T_{15} and T_{20} would either be improved or at least maintained when the collected plant parts were combined. However, when combining plant parts the MIC remained unaltered throughout the test protocol. It is as yet not clear why there was a loss in efficacy at T_{15} and T_{20} when leaves were added.

The uniqueness of this study is emphasised when it is compared to relevant South African studies. Ndhlala et al. (2009) and Mulaudzi et al. (2011) focussed on *A. chabaudii* and *A. barberae*; and reported relatively poor antimicrobial activity of aqueous extracts against *S. aureus* (MIC range: 3.125–6.25 mg/ml). These authors employed root (Ndhlala et al., 2009; Mulaudzi et al., 2011) and leaf extracts (Ndhlala et al., 2009) prepared from dried material which was added to cold water. The only studies on *A. marlothii* subsp. *marlothii* relevant to ours are the reports by Luseba et al. (2007) and Naidoo et al. (2013). Even though Naidoo et al. (2013) did not use exactly the same pathogens as we did, *C. albicans* and *N. gonorrhoea* were included. This study reported very poor activity (MIC ≥ 16 mg/ml) against *C. albicans* and *N. gonorrhoea* when aqueous extracts were used. Further investigation of their preparation method indicated that the aqueous extraction was done over 24 h with powdered plant material; as no specific mention was made to the temperature of the water, it is fair to argue in favour of room temperature. The significantly better antimicrobial activity reported in our study on aqueous extracts positively reflects on the following; the use of fresh plant material which seems to be superior to dried material, and the limited boiling of plant material. However, in contrast to the traditional way of preparing extracts with water, both Luseba et al. (2007) and Naidoo et al. (2013) indicated that the use of organic solvents such as dichloromethane and methanol can enhance the antimicrobial activity of an extract. It is therefore recommended that the combination of roots and leaves be further pursued using a test panel of solvents covering the polar to non-polar range. It is recognised that suggestions regarding the use of roots is not entirely within the

framework of conservation and/or sustainable utilisation; however, collection of roots can be achieved without imposing an excessive risk to the survival of the plant, and ultimately the species.

8.5 CONCLUSION

As far we can ascertain, the current work is the first to report on the antimicrobial efficacy of *A. marlothii* subsp. *marlothii* aqueous extracts as employed by the Bapedi in the treatment of STIs. The results reveal that these aqueous extracts are not without merit when used in the treatment of STIs. To an extent, results such as these, lend credibility to its use in traditional medicine. It also emphasises the possibility of further pharmacological assessment in the search of novel leads to address the multidrug resistant issue.

CHAPTER 9

Antimicrobial activity of *Ziziphus mucronata* crude aqueous multi-extracts

In vitro antimicrobial potential of aqueous multi-extracts containing *Ziziphus mucronata* subsp. *mucronata* as a common denominator: Does boiling time affect its efficacy?

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ABSTRACT

Ethnopharmacological relevance: The therapeutic use of plants in the treatment and prevention of diseases is currently a key focus point worldwide. In South Africa approximately 3000 plant species are used, by more than 200 000 traditional healers, to treat a wide array of diseases, including sexually transmitted diseases. The focus of this study lie in the fact that *Ziziphus mucronata* was often mentioned in multi-plant decoctions used to treat STIs.

Aim of study: This study was conducted to verify the *in vitro* antimicrobial activity of specifically identified plant species as well as the impact of boiling time on such activities.

Materials and methods: Using the micro-dilution method for crude aqueous extracts prepared from fresh plant material, the inhibitory potential against *Neisseria gonorrhoea*, *Proteus vulgaris*, *Staphylococcus aureus* and *Candida albicans* was investigated.

Results: Inhibitory effects varied from moderate to poor, with only a few noteworthy (MIC < 1 mg/ml) responses. *Staphyllococcus aureus* and *C. albicans* were the most susceptible STI related pathogens. However, limited excellent antibacterial activity was observed against *N. gonorrhoea* and *P. vulgaris*. *Ziziphus mucronata* exhibited the best overall antimicrobial activity of the single extracts; with *T. terrestris*

performing the worst of all. Combining *H. hemerocallidea* and *T. terrestris* with *Z. mucronata* often resulted in improved antimicrobial activity.
Conclusion: The combination of plants as well as extended boiling time did alter the antimicrobial activity of the crude extracts.

Keywords: Antimicrobial activity, Bapedi traditional healers, *Candida albicans*, *Hypoxis hemerocallidea*, *Neisseria gonorrhoea*, *Proteus vulgaris*, sexually transmitted infections, *Tribulus terrestris*, *Ziziphus mucronata*.

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9.1 INTRODUCTION

The role of traditional medicine in primary health care encapsulates many aspects. One such focus area relates to ethnobotanical studies of traditional medicine in local communities as well as the ethnopharmacological research into the bioactivity of traditionally used medicinal plants (Vandebroek, 2013). According to the WHO (2008), “traditional medicine” is a complex concept that involves indigenous knowledge, skills and practices based on theories, beliefs and experiences as dictated within a specific cultural environment. Plants have always been an essential component of this traditional medicine system (Fang et al., 2005); and they remain relevant as therapeutics in developing countries (Sokmen et al., 1999). Therefore, investigating traditionally used plants in the search of novel therapeutic compounds is still an applicable practice (Tshikalange et al., 2005).

Ziziphus mucronata subsp. *mucronata* (*blinkblaar-wag-‘n-bietjie* in Afrikaans, *buffalo-thorn* in English), occurs in a wide variety of habitats, which can range from semi-desert to forest (Van Wyk et al., 2011). It is a small to medium-sized tree with a wide, spreading crown and rough, grey-brown bark. Thorns are paired; the one straight, the other curved, and they are usually present on twigs. The characteristic shiny green leaves have three main veins arising from the base, and the upper half

of the leaf's margin is toothed (Van Wyk et al., 2009). Its prominence as a multipurpose medicinal species, as well as the scientific assessment of some of its ethnobotanical uses are summarised in Tables 9.1 and 9.2.

The emergence of multiple-drug resistant pathogens has become a major cause of concern in the treatment of infectious diseases (Gibbons, 2005). The threat that these resistant pathogens pose to human health has prompted investigations into the discovery of new antimicrobials. At the centre of these investigations are plant derived compounds, as plants are rich sources of bioactive compounds. Crude plant extracts and their bioactive constituents have been known to possess biological potential; especially antibacterial (Green et al., 2010; Mulaudzi et al., 2011) and antifungal (Shai et al., 2009; Amoo et al., 2011) activities. Therefore, any particular substance isolated from such crude extracts that can inhibit pathogens, and have very low levels of toxicity towards host cells, could be favourably considered for the development of novel antimicrobial drugs (Bajpai et al., 2005).

Traditionally, crude extracts prepared from plants, can be used either independently or as combinations. It is also possible that a combination extract can contain different parts of the same plant (York et al., 2011). The most common preparation medium is water; some lay people add the plant material to cold water and bring the mixture to boil (York et al., 2011), whilst others add the plant material to boiling water and then continue to boil it for a fixed period (Erasmus et al., 2012), or until a specific colour change is observed (Semenya, 2012). Even though modern laboratory practices question the efficacy of aqueous extracts; it is still extensively being used by traditional healers. More often than not aqueous extraction procedures performed in laboratories do not focus on mimicking the traditional ways per se, but rather on the fact that water is used as a polar solvent.

The use of multi-plant decoctions to treat STIs is well documented (Erasmus et al., 2012; Semenya, 2012). In that regard certain species feature more often than others. *Ziziphus mucronta*, a species regularly used by Bapedi traditional healers, is implicated as a key species in the preparation of STI remedies (Erasmus et al., 2012; Semenya, 2012). This creates the notion whether this species should be considered as a baseline species, amongst the Bapedi, in the control of STIs.

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Antimicrobial activity of *Ziziphus mucronata* crude aqueous multi-extracts

Therefore, the aim of this chapter was to investigate the antimicrobial potential of aqueous extracts with *Z. mucronata* as a common denominator; with specific emphasis on the contribution of boiling time.

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Antimicrobial activity of *Ziziphus mucronata* crude aqueous multi-extracts

Table 9.1: A summary of the traditional medicinal uses including a record of the various plant parts used in aqueous preparations of *Ziziphus mucronata*.

Traditional use	Plant part used	References
Sores, ulcers, skin inflammation or infections, boils, sores, glandular swelling, gonorrhoea, diarrhoea, dysentery, expectorant, emetic, chest problems	L, R, B	Watt and Breyer-Brandwijk (1962), Hutchings et al. (1996), Iwalewa et al. (2007), Luseba et al. (2007), Appidi et al. (2008), Van Wyk et al. (2011)
Candida infections	RB	Runyoro et al. (2006)
Anthelmintic	F	Von Maydell (1990)
Boils, abdominal pains, female infertility and wounds	F, L, R	Maroyi (2011)
Blenorrhagia, female sterility, dysmenorrhoea and wounds	RB, SB	Mpiana et al. (2008)
Obesity, against hunger feeling, sedative, tonic, cold, lumbago, tumour, chlamydia and gonorrhoea, toothache, scrofula, stomach ache, constipation, muscular pain, menorrhagia and infertility	R	Palmer and Pitman (1972), Arnold and Gulumian (1984), Iwu (1993), Johnson (1999), Bruschi et al. (2011), Nadembega et al. (2011), Semenya (2012)
Coughs, chest ailments and fever	B	Mativandlela et al. (2008)
Fertility enhancement, sores and burns	L, R	Van der Merwe et al. (2001), Mabogo (1990).
Pain, enteric conditions, malaria and eye diseases	L	Kudi and Myint (1999), Lindsey et al. (1999), Van Wyk et al. (2011)

Plant part used: leaves = L, roots = R, bark = B, root bark = RB, stem bark = SB and fruit = F.

Table 9.2: Scientific assessment of *Ziziphus mucronata* subsp. *mucronata* extracts as recorded in the literature.

Plant part	Solvents	Activity / use assessed	Pathogens / organisms tested	References
B	DCM, MeOH	Anti-bacterial, anti-inflammatory, mutagenic	*Pa, Sa, Ec*	Luseba et al. (2007)
		Anti-oxidant		Frum (2006)
B	A		*Mt*	Green et al. (2010)
L	H_2O, MeOH			Bessong et al. (2005)
R, T	DCM, MeOH	Genotoxicity		Elgorashi et al. (2003)
L, R, T	DCM, MeOH	Mutagenic and anti-mutagenic		Verschaeve and Van Staden (2008)
SB	H_2O, MeOH	Antigungal	*Ca*	Gundidza (1986)
F, T, W	H_2O, DCM+MeOH	Anti-nematodal	*Ce*	Waterman et al. (2010)
L	DCM, DCM + MeOH, MeOH, H_2O	Antiplasmodial	*Pf*	Clarkson et al. (2004)
R		Anthelmintic	*Sh*	Aremu et al. (2012)
R	MeOH, Ethyl acetate	Acetylcholinesterase inhibition and oxidative		Adewusi and Steenkamp (2011)
B, R	H_2O	Antibacterial	*Pm, Pa, Sa, Ec*	Adamu et al. (2005)
RB, SB	H_2O, EtOH	Antisickling		Mpiana et al. (2008)
L	EtOH	Antibacterial	*Ms, Mt*	Mativandlela et al. (2008)

L, B	H, MeOH, H_2O	Anthelmintic, antibacterial, cytotoxicity	*Pa, Sa, Ec, Ef*	McGaw et al. (2007)
L	H_2O, EtOH	Prostaglandin-synthesis inhibition		Lindsey et al. (1999)
	EtOH	Uterine smooth muscle contraction		
L	MeOH	Antiviral, cytotoxic	Poliovirus, astrovirus, herpes simplex virus 1, equine herpes simplex virus, bovine parvovirus and canine parvovirus	Kudi and Myint (1999)
R	MeOH	Cytotoxicity		Kamuhabwa et al. (2000)
L	H_2O, EtOH	Prostaglandin-synthesis inhibitors		Jäger et al. (1996)
L	H_2O, EtOH	Anti-amoebic		McGaw et al. (2000)

Plant part used: leaves = L, roots = R, bark = B, root bark = RB, stem bark = SB, fruit = F, twigs = T and wood = W. Solvents used: dichloromethane = DCM, methanol = MeOH, ethanol = EtOH, hexane = H, aqueous = H_2O, acetone = A.

Pathogens/organisms: *Caenorhabditis elegans* = Ce, *Candida albicans* = Ca, *Enterococcus faecalis* = Ef, *Escherichia coli* = Ec, *Mycobacterium smegmatis* = Ms, *Mycobacterium tuberculosis* = Mt, *Plasmodium falciparum* = Pf, *Proteus mirabilis* = Pm, *Pseudomonas aeruginosa* = Pa, *Schistosoma haematobium* = Sh, *Staphyllococcus aureus* = Sa.

9.2 MATERIALS AND METHODS

9.2.1 Sample collection

Plant material (root and corm) were collected in February 2013, from Ga-Mamadila village (Figure 9.1), Capricorn District, Limpopo Province (S 23°47'10.0"; E 29°13'42.8"). Voucher specimens were collected and deposited at the Larry Leach Herbarium (UNIN) of the University of Limpopo. The roots of *Ziziphus mucronata* (n = 3) and *Tribulus terrestris* (n = 6), and the corms of *Hypoxis hemerocallidea* (n = 7) were collected where the species grew in close proximity of each other. This was done to ensure that samples were collected from species growing in the same strata, thus minimizing the effect of soil composition on the phytochemical profile.

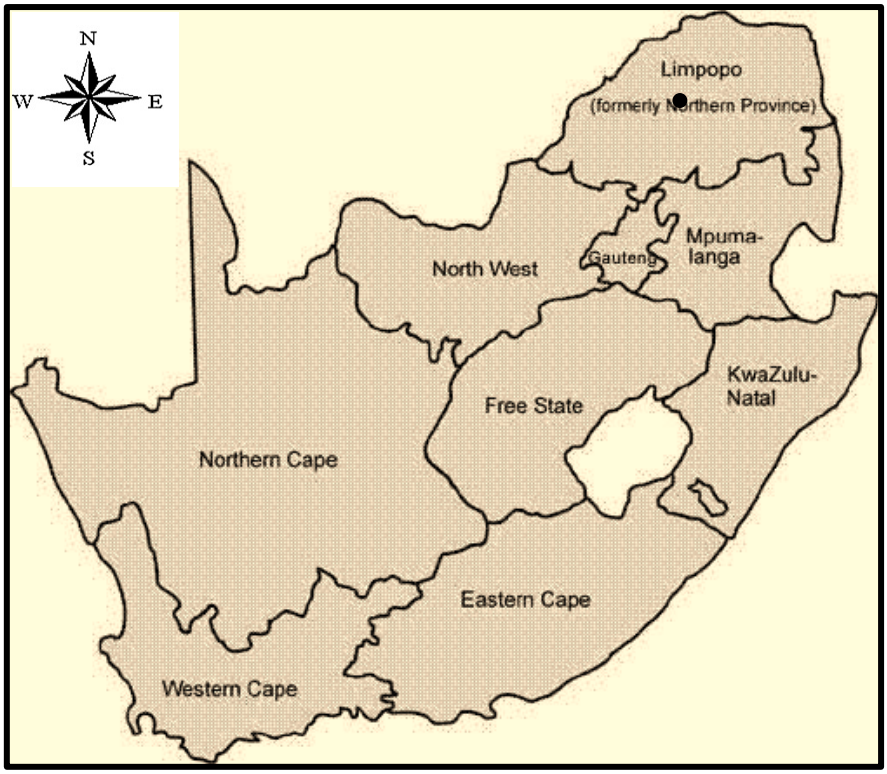

Figure 9.1. Map of South Africa, showing the study area (Ga-Mamadila village) within the Limpopo Province.

9.2.2 Extraction

9.2.2.1 Preparation of plant extracts

Only fresh subterranean plant material was used in this study. The yield for all of these species were very small, thus it was decided to combine the fresh material collected from species growing in close proximity of each other. After collection the roots and tubers were cut into smaller pieces. The following weights of fresh root / corm material was added separately to boiling tap water; 240 g of *Z. mucronata*, 850 g of *H. hemerocallidea*, 14 g of *T. terrestris*, and 100 g and 114 g respectively for the *Z. muconata* / *H. hemerocallidea* combination. The disparity in the *Z. mucronata* (57.4 g) and *T. terrestris* (12 g) combination relates to the fact that *T. terrestris* had a very low yield as a limited number of roots could be collected in this specific area, and the roots were very small. At specific time intervals (T_{00}, T_{05}, T_{10}, T_{15} and T_{20} minutes) 500 ml of the boiling liquid was removed from the pot. These volumes were separately filtered through Whatmann No. 1 filter paper, and stored in appropriately labelled glass containers. Filtered crude extracts were frozen; where after the water was removed via freeze drying (VIRTIS, United Scientific (PTY) Ltd.). The dried, concentrated extracts were used to prepare 50 mg/ml stock solutions to be used in the micro-dilution assays.

Time 0 (T_{00}) was considered the removal of a 500 ml volume within 15–20 seconds after adding the plant material to the boiling water. The other samples were collected at 5 minute intervals succeeding T_{00}.

9.2.3 Antimicrobial activity

9.2.3.1 Bacterial and fungal cultures

The choice of pathogens for investigation was based on STIs commonly associated with an abnormal urethral discharge. These were *N. gonorrhoeae* (ATCC 49226) and *Proteus vulgaris* (ATCC 6380) two Gram-negative bacteria, and *Candida albicans*

(ATCC 10231) a Gram-positive fungus. *Staphylococcus aureus* (ATCC 25923), a Gram-positive bacterium was also included in the test protocol. Where no reference strains were available clinical strains were used.

Neisseria gonorrhoeae was obtained from ATCC as KWIK-STIK® plus microorganism. The bacteria were inoculated onto chocolate agar (Oxoid GC agar base) which was supplemented with 2% (W/V) of haemoglobin and 1% (v/v) of Vitox supplement; where after it was incubated for almost 48 h at 37 °C in 5% CO_2. A number of colonies of pure culture were collected from an overnight culture and suspended in 5 ml Mueller-Hinton (MH) broth. The turbidity of this cell suspension, at 540 nm, was adjusted by adding either MH broth or organism as required, until the turbidity of the suspension was equivalent to that of a 0.5 McFarland $BaSO_4$ standard to approximately 108 CFU/ml.

Candida albicans, *P. vulgaris* and *S. aureus* were inoculated onto nutrient broth. With the exception of *C. albicans* (48 h), the incubation period was 24 h at 37 °C. The turbidity of all suspensions was adjusted using the McFarland standard.

9.2.3.2 Microdilution method

The minimum inhibitory concentration (MIC) values were determined using the micro-titre plate dilution method adapted from Eloff (1998). In general all assays were performed in triplicate; the only exception being some of the T_{00} samples, where the yield of active compounds was so small that only duplicate assessments could be performed. Stock solutions of the plant extracts were made up to 50 mg/ml using distilled water; dimethyl sulfoxide (DMSO) was not required as the concentrates were initially prepared from aqueous solutions. Nutrient broth (100 µl) was pipetted into all wells of the micro-titre plate. Thereafter, stock solutions (100 µl) of each of the prepared extracts were transferred into the first row (row A) of the micro-titre plate. Serial dilutions were performed, starting at 12.5 mg/ml and ending at 0.09 mg/ml. A standard volume of 100 µl of culture was added to all 96 wells. The micro-titre plates were then incubated at 37 °C for a period of time, which was similar to those periods used when preparing the different colonies. Following the period of

incubation, 40 µl of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) (Sigma Chemical Company, St. Louis, MO) was added to all wells. After addition of INT the micro-titre plates were incubated for 30 minutes at 37 °C. The INT viability indicator was added to determine visually where microbial growth changed the colour of the solution; where a red-pink colour indicated viable bacteria and clear-yellow indicated inhibition. The inclusion of negative controls ensured that the relevant nutrient or MH broth was not contaminated. The broad spectrum antibiotic Ciprofloxacin was used as a positive control; serial diluted from 0.25 mg/100µl.

9.3 RESULTS

The antimicrobial MIC values of the crude extracts of the three medicinal plants, and their respective combinations, used by the Bapedi traditional healers are presented in Tables 9.3 and 9.4. In agreement with Gibbons (2005), the current study considered MIC values ≤1 mg/ml as an indication of excellent antimicrobial activity.

9.4 DISCUSSION

Selection of these species, and the subsequent combinations, was done according to a more comprehensive unpublished report, which investigated the ethnobotany of the Bapedi in the Limpopo Province, South Africa. All extracts were prepared using water; and boiled according to the range of boiling times observed.

9.4.1 *Ziziphus mucronata* and *Hypoxis hemerocallidea*

Existing ethnobotanical literature (Table 9.1) supports the fact that aerial and subterranean parts of *Z. mucronata* are used for medicinal purposes. However, information regarding the antimicrobial activity of *Z. mucronata* is limited. Two previous studies reflected on the antibacterial efficacy of aqueous extracts. McGaw et al. (2007) assessed bark and leaf extracts using hexane, methanol and water as solvents. Extract preparation was based on adding 2 g of dried, powdered plant

Table 9.3: *In vitro* antimicrobial activity of *Ziziphus mucronata* and *Hypoxis hemerocallidea*.

Extract	Time (min)	Pathogens tested			
		N. gonorrhoea	*P. vulgaris*	*S. aureus*	*C. albicans*
		MIC (mg/ml)			
Ziziphus mucronata	0	1.56	1.56	------	1.56
	5	3.12	3.12	6.25	1.56
	10	1.56	1.56	**0.39**	1.56
	15	1.56	1.56	**0.19**	1.56
	20	1.56	1.56	>12.50	1.56
Hypoxis hemerocallidea	0	1.56	**0.78**	3.12	**0.78**
	5	3.12	1.56	1.56	1.56
	10	6.25	1.56	1.56	1.56
	15	6.25	1.56	6.25	1.56
	20	12.5	3.12	3.12	3.12
Ziziphus mucronata and Hypoxis *hemerocallidea*	0	1.56	12.50	**0.19**	**0.78**
	5	1.56	12.50	**0.19**	**0.78**
	10	1.56	3.12	**0.19**	1.56
	15	1.56	3.12	**0.39**	1.56
	20	1.56	6.25	12.50	1.56
Ciprofloxacin	<0.09 mg/ml against all tested pathogens				

---- indicates that no crude extracts was available to be tested.

material to 20 ml of the solvent and shaking it vigorously for 20 min. They found that both bark and root aqueous extracts showed poor antibacterial activity (>12.5 mg/ml) against *S. aureus*. Adamu et al. (2005), macerated 50 g of dried, powdered root bark in 250 ml of water for four days. Employing the agar-well diffusion method, they reported strong inhibitory activity against *Proteus mirabilis* and *S. aureus*. In the

Table 9.4: *In vitro* antimicrobial activity of *Ziziphus mucronata* and *Tribulus terrestris*.

Extract	Time (min)	Pathogens tested			
		N. gonorrhoea	*P. vulgaris*	*S. aureus*	*C. albicans*
		MIC (mg/ml)			
Ziziphus mucronata	0	1.56	1.56	------	1.56
	5	3.12	3.12	6.25	1.56
	10	1.56	1.56	**0.39**	1.56
	15	1.56	1.56	**0.19**	1.56
	20	1.56	1.56	>12.50	1.56
Tribulus terrestris	0	6.25	>12.50	>12.50	12.50
	5	6.25	>12.50	>12.50	12.50
	10	6.25	>12.50	>12.50	12.50
	15	6.25	>12.50	12.50	12.50
	20	6.25	>12.50	12.50	12.50
Ziziphus mucronata and *Tribulus terrestris*	0	3.12	12.50	------	>12.50
	5	6.25	1.56	**0.39**	3.12
	10	**0.78**	1.56	**0.78**	1.56
	15	1.56	1.56	**0.39**	3.12
	20	3.12	1.56	6.25	1.56
Ciprofloxacin	<0.09 mg/ml against all tested pathogens				

---- indicates that no crude extracts was available to be tested.

present study, most of the aqueous extracts exhibited good antimicrobial activity (MIC = 1.562 mg/ml) against *P. vulgaris*, *N. gonorrhoea* and *C. albicans*. In comparison to the preparation protocol of McGaw et al. (2007), the boiling of fresh plant material clearly yielded better antibacterial activity against *S. aureus*. It is noteworthy that 10 and 15 minutes of boiling resulted in excellent antibacterial activity (MIC 0.195–0.390 mg/ml), undeniably indicating the mobilisation of bioactive compounds with a strong inhibitory activity against *S. aureus*. The predominantly consistent response (MIC = 1.562 mg/ml) by *N. gonorrhoea*, *P. vulgaris* and *C.*

albicans was unexpected. This is based on the fact that *S. aureus* in its varying inhibitory response clearly indicated that the composition of the extracts changed asboiling time progressed, yet the response by the other pathogens remained relatively consistent. A plausible explanation involves the decreased susceptibility of the Gram-negative bacteria as compared to *S. aureus*, and the possibility that the principal compound responsible for the Gram-negative response did not change significantly and was accessible throughout the boiling period.

The antibacterial activity of *H. hemerocallidea* have been scrutinised in previous investigations. Katerere and Eloff (2008), in their conservation quest to proof that leaves can be substituted for corms, found that dried corms were far more effective than leaves when used against *S. aureus*. However, even though their MIC values were 0.31 mg/ml for acetone and 0.63 mg/ml for ethanol, they did not use an aqueous extract nor compare fresh corms with dried corms. More recently Naidoo et al. (2013) reported on the efficacy of aqueous and dichloromethane:methanol (1:1) extracts against various pathogens, including *C. albicans* and *N. gonorrhoea*. With the exception of the activity of the aqueous extract against *N. gonorrhoea* (MIC 0.5 mg/ml), none of the remaining extracts succeeded in significantly (MIC < 1.0 mg/ml) inhibiting these pathogens. The fact that our aqueous extracts did not achieve the same level of efficacy as that reported by Naidoo et al. (2013), can probably be attributed to the differences in preparation procedures. It seems as if the maceration of dried plant extracts for a period of 24 h did indeed create a crude extract that contained compounds that could not be accessed during our boiling intervals. It is also possible that the boiling period either destroyed the principal constituents, or that it was not long enough to release sufficient concentrations of it to induce a noteworthy effect.

No other records, except our observations among the Bapedi, could be located regarding the simultaneous use of *Z. mucronata* and *H. hemerocallidea* against STI pathogens. However, *H. hemerocallidea* have been used in combination with other species in the treatment of urinary tract infections (Drewes et al., 2008) and as an anti-HIV agent (Pooley, 2005). This combination performed on par with *Z. mucronata* singularly when tested against *N. gonorhoea*. However, that being said, synergism

was observed for the boiling period T_{05} when the MIC was improved from 3.125 mg/ml to 1.562 mg/ml. In contrast to this observed synergistic effect, an antagonistic response was observed against *P. vulgaris* when these species were combined (Table 9.3). This variation in the response of *N. gonorrhoea* and *P. vulgaris* illustrates the unique dissimilarity in susceptibility amongst Gram-negative bacteria. The overall antibacterial performance of these plant species against *S. aureus* was significantly enhanced when they were combined. Nevertheless, the synergistic effect observed for the boiling period $T_{00–10}$, gradually faded. The most prominent change in efficacy against *C. albicans* was observed for T_{05}. Even though all three extracts exhibited potential against this pathogen, the most significant antifungal responses were observed early on, in the boiling of the combined plant material collected from *Z. mucronata* and *H. hemerocallidea*. The bioactive composition of the combined extract remained unaltered at the commencement of the boiling protocol and was sustained up to T_{05}. Except for the decrease in efficacy observed for *P. vulgaris*, this study found that the combination of these two plant species showed antimicrobial potential and needs further pharmacological assessment.

9.4.2 *Ziziphus mucronata* and *Tribulus terrestris*

The antimicrobial activity of *T. terrestris* seems to relate to the plant part used, where it originated as well as the type of solvent used (Hussain et al., 2009). It is believed that the antimicrobial activity is determined by the presence of spirosaponins (Bedir and Khan, 2000), which are glycosides of spirostane triterpenoid sapogenins (Polya, 2003). Similar to previous studies (Usman et al., 2007; Mohana et al., 2008; Hussain et al., 2009), the current study didn't find significant antimicrobial activity for *T. terrestris* against *S. aureus*, *C. albicans* and *Proteus* spp.; irrespective of plant part or solvent used. The best inhibitory response in the current study, even though not noteworthy (MIC = 6.25 mg/ml), was against *N. gonorrhoea*. To the best of our knowledge, this is the first assessment of the antibacterial activity of *T. terrestris* against this pathogen. It is currently not clear why the MIC values remained relatively unchanged for all pathogens, throughout the boiling protocol; especially if these

values are compared to those of the combined extracts. This warrants further elucidation.

Combining *Z. mucronata* with *T. terrestris* exhibited antimicrobial inhibitory responses that showed both antagonistic and synergistic interactions. A general decrease in activity was observed for *C. albicans*; emphasising the fact that the addition of *T. terrestris* dramatically influenced the activity exhibited by *Z. mucronata*. Noteworthy activity of the combination against *S. aureus*, was detected in the boiling period $T_{05–15}$; the best overall antimicrobial activity observed in this study. It is evident that *Z. mucronata* plays a key role in the $T_{10–15}$ boiling period, buffering the poor inhibitory activity observed for *T. terrestris*. However, the synergistic response detected at T_{05} and T_{20} is difficult to explain in terms of species dominance. It is possible that at these boiling times, the individual plant species had sub-threshold levels of certain bioactive compounds that was subsequently increased when the plants were combined, thereby improving the activity against *S. aureus*. Good activity was noted for *Z. mucronata* against *P. vulgaris*; singularly and in combination; and seemingly independent of boiling time. Of interest is the antagonistic (T_{00}) and synergistic (T_{05}) effects recorded. The good activity of *Z. mucronata* was completely overshadowed by the poor activity of *T terrestris*, when the species were combined, resulting in an MIC of 12.5 mg/ml. In contrast to this, the synergistic inhibitory response at T_{05} indicates the increased availability of some bioactive compounds. It is reasonable to surmize that these compounds were mobilised from *T. terrestris* after 5 minutes of boiling. *Neisseria gonorrhoea*, a fastidious Gram-negative bacterium, is of particular interest in this study; it is one of the most prominent pathogens associated with an abnormal urethral discharge. In the present study relatively poor inhibitory activity against this pathogen was noted for almost all of the aqueous extracts. However, excellent activity was observed when the plants were combined and boiled for 10 minutes. This response was gradually lost with extended boiling times. It is reasonable to argue in favour of increased mobilisation of specific bioactive compounds, regarding the T_{10} response. However, this phenomenon requires further investigation, using a wider spectrum of

solvents with different polarities; as well as high performance liquid chromatography to clarify the bioactive profiles of these extracts.

9.5 CONCLUSIONS

The current study, based on establishing antimicrobial potential, found no distinct reaction patterns to support the inclusion of *Z. mucronata* as a common denominator. This creates the impression that its inclusion can be attributed to the alleviation of symptoms through mechanisms other than the inhibition or killing of bacterial strains. It is possible that its continued use by Bapedi traditional healers, to alleviate the abnormal urethral discharge, is most probably the result of an anti-inflammatory contribution.

When assessing the impact of boiling time on antimicrobial efficacy, evidence suggests that it can significantly alter the bioactive profiles. The extent of this alteration is not clear and further investigation is required. Notable inhibitory activity suggests that extended boiling time can indeed increase or maintain such activities. These results partially validate the traditional use, and preparation of plant-specific aqueous extracts, in the treatment of STIs.

CHAPTER 10

Antimicrobial activity of *Catharanthus roseus* crude aqueous extracts

In vitro assessment of the antimicrobial efficacy of aqueous root extracts of *Catharanthus roseus*: Bapedi boiling preferences

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ABSTRACT

Catharanthus roseus is widely used by the Bapedi in the Limpopo Province, South Africa, to treat gonorrhoea. However, scientific validation regarding the antimicrobial efficacy of this species, based on traditional preparation protocols, is lacking. The current study was designed to investigate the *in vitro* antimicrobial activity of crude aqueous extracts prepared from the roots of *C. roseus*. Aqueous extracts, prepared from the roots of *C. roseus*, were subjected to the boiling protocols favoured by the Bapedi. Antimicrobial activity was determined using the minimum inhibitory concentration (MIC) assay against the following STI-related pathogens: *Candida albicans* (ATCC 10231), *Neisseria gonorrhoea* (ATCC 49226), *Proteus vulgaris* (ATCC 6380), and *Staphylococcus aureus* (ATCC 25923). All pathogens tested, with the exception of *N. gonorrhoea*, exhibited low levels of susceptibility (MIC 6.25–12.5 mg/ml) towards the various aqueous extracts used. However, all these extracts showed noteworthy inhibitory potential against *N. gonorrhoea* (MIC 0.781 mg/ml).

The present study scientifically validated the traditional use of crude *C. roseus* root extracts, by the Bapedi, in the treatment of *N. gonorrhoea* related infections.

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The exact contribution of boiling time towards the bioactive profiles could not be assessed and requires further investigation.

Keywords: Bapedi, Boiling, *Candida albicans*, *Catharanthus roseus*, crude extracts, *Neisseria gonorrhoea*, *Proteus vulgaris*.

10.1 INTRODUCTION

Primary health care needs, especially in rural areas of developing countries, more often than not rely on the use of natural products. According to the World Health Organization (WHO, 2011a) estimates are that 70–95% of the world's population, in developing countries, depend primarily on plants to address this need. In addition, the elevated demand for inexpensive medicines, the rise in unemployment as well as the increased incidence in infectious diseases, and the concomitant multi-drug resistance to pathogens involved makes the use of natural products an attractive alternative. In traditional medicine the treatment protocols, encompassing aspects such as plant identification, extract preparation and administration, are based on extensive knowledge and expertise amongst the members of local communities (Shai et al., 2008; Street et al., 2008). Therefore, instead of depending on trial and error, as in the random screening of species, traditional knowledge assists scientists in identifying plants that may be medicinally useful (Cox and Balick, 1994).

Most modern-day plant-derived drugs were originally used in traditional medicine, and through scientific validation became part of the Western armament against diseases. One of the greatest successes in modern medicine is the use of compounds extracted from *Catharanthus roseus* (L.) G.Don (Apocynaceae). It is a perennial pan-tropical ornamental and naturalised species (Gericke, 2011), which was originally noticed for its impact on the glucose metabolism, hence its application in the treatment of diabetes mellitus (Ohadoma and Michael, 2011). However, of far greater relevance was the discovery of the various dimeric monoterpenoid indole alkaloids with their anti-mitotic activity, which revolutionised cancer chemotherapy (Jordan and Wilson, 2004). Aerial parts, such as leaves and stems are rich sources

of the indole alkaloids vinblastine and vincristine, both indispensable in cancer treatment (Jaleel et al., 2007a). Nevertheless, this species has a far wider medicinal application than chemotherapy alone (Table 10.1).

Interest in the medicinal value of this species is immense and has resulted in the appearance of more than 2000 publications and approximately 295 patents, since 1950 (Govindaraji, 2007). It is therefore ironic that, within the context of increased multi-drug resistance from pathogens, very few of these studies actually deal with the antimicrobial potential of *C. roseus*. A number of ethnobotanical studies (Table 10.1) emphasised the use of various plant parts in the treatment of sexually transmitted infections (STIs). However, scientific validation regarding the antimicrobial efficacy against specific pathogens, implicated as causative agents in STIs, are lacking. It was previously reported by Erasmus et al. (2012) that the Bapedi from the Limpopo Province, South Africa, use the roots from *C. roseus* in the exclusive treatment of gonorrhoea; or possibly other related infections that causes a characteristic abnormal urethral discharge. It was therefore the aim of this chapter to investigate the antimicrobial efficacy of aqueous root extracts, within the confines of the preferred Bapedi preparation methods.

10.2 Materials and Methods

10.2.1 Plant collection

Plant materials were collected in February 2013, from Ga-Mamadila village, Capricorn District, Limpopo Province (S 23°47’15.2”; E 29°13’32.3”). Voucher specimens (Cr1, 2, 3, 4 and 5) were collected and deposited at the Larry Leach Herbarium (UNIN) of the University of Limpopo. Subterranean parts were collected

CHAPTER 10

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Table 10.1: Summary of the traditional and modern medicinal applications of *Catharanthus roseus* as recorded in the literature.

Parts used	Material	Application		Therapeutic uses / activity	Preparation / extraction	Pathogens tested	Reference
		T	M				
L	Fresh		X	Immuno-enhancing	H_2O		Patra et al. (2010)
L	Fresh		X	Antibacterial potential	EtOH, MeOH, A	*Sa, Pa, Bs, St*	Patil and Gosh (2010)
L			X	Anti-cancer, anti-tumour			Levêque et al. (1996), Kruczynski et al. (1998), Duffin (2000), Anna and Bridget (2001), Montbriand (2004), Bennouna et al. (2005)
F			X	Skin growths			Hutchings (1996)
R		X		Sexually transmitted infections	H_2O		Bruschi et al. (2011)
		X		Abortifacient			Zaguirre (1944, as cited in Kumar et al., 2012)

L	Dried		X	Antibacterial	EA, A, MeOH	*Kp, Ah, Ea, Ec, Bc*	Kamaraj et al. (2012)
			X	Diabetes mellitus model	Vindolene		Yao et al. (2013)
L, T	Dried		X	Antidiabetic	H_2O, DCM:MeOH		Van de Venter et al. (2008), Malviya et al. (2010)
L		X		Nosebleeds, bleeding gums, mouth ulcers, sore throat, gastritis, enteritis, diarrhoea			Rath et al. (2012)
L	Dried		X	Antibacterial	H_2O, EtOH	*Cf*, *Pa*, *Ab*, *Pm*, *Pv*, *Ko*	
L	Dried		X	Anthelmintic	EA, A, MeOH	*Haemonchus contortus*	Kamaraj and Rahuman (2011)
L			X	Antiplasmodial, cytotoxic	H_2O, MeOH		Gathirwa et al. (2007)
L, S, F, R	Fresh		X	Antibacterial	EtOH, MeOH	*Ec*, *Pa*, *Sm*, *Sti*, *Sa*, *Sp*, *Bc*, *Bs*	Ramya et al. (2008)
A			X	Liver enzymes	MeOH		Usia et al. (2006)

F, L	Fresh		X	Antituberculosis	MeOH	*Mt*	Mohamad et al. (2011)
F	Fresh		X	Wound healing	EtOH		Nayak et al. (2007; 2006)
		X		Urinary ailments			Chellappandian et al. (2012)
L, S, R, F	Dried		X	Antibacterial	EtOH, MeOH	*Bs*, *Ec*, *Sa*, *Sti*, *Pa*	Govindasamy and Srinivasan (2012)
R		X		Gonorrhoea			Erasmus et al. (2012); Semenya et al. (2012)
L	Dried		X	Antimalarial	H_2O, MeOH, EA	*Anopheles stephensi*, *Culex quiquefasciatus*	Subarani et al. (2013)
R	Dried		X	AchE Inhibitors	H_2O		Pereira, Ferreres, et al. (2010)
E	Dried		X	Cytotoxicity	C		Wang et al. (2013)
R	Dried		X	Antioxidant	H_2O, MeOH		Pereira, Faria et al. (2010)
L	Dried		X	Antihyperlipidemic, antioxidative			Chauhan et al. (2012)
E	Dried		X	Antibacterial	DCM:MeOH	*Kp, Ec, Pa, Pv, Bc, Bs, Sa*	Ibrahim et al. (2011)

L	Fresh		X	Hypoglycaemic	MeOH		Nammi et al. (2003), Ohadoma and Michael (2011)
L	Dried		X	Antifungal	PE, DE, C	*Fo, Bca, Aa*	Roy and Chatterjee (2010)
L	Dried		X	Genotoxic effects	DCM, MeOH		Elgorashi et al. (2003)
L			X	Mutagenicity, antimutagenicity	DCM, MeOH		Verschaeve and Van Staden (2008)
F, L		X		Non-communicable diseases	H_2O		Chintamunnee and Mahomoodally (2012)

Application: T = traditional, M = modern; Parts used: L = leaves, F = flowers, R = roots, T = twigs, S = stems / stalks, A = aerial parts, E = entire plant. **Extraction:** H_2O = aqueous, EtOH = ethanol, MeOH = methanol, A = acetone, EA = ethyl acetate, DCM = dichloromethane, C = chloroform, PE = petroleum ether, DE = diethyl ether. **Pathogens:** *Acinetobacter baumanii* = Ab, *Aeromonas hydrophila* = Ah, *Alternaria alternata* = Aa, *Bacillus cereus* = Bc, *Bacillus subtilis* = Bs, *Botrytis cinerea* = Bca, *Critobacter freundii* = Cf, *Enterobacter aerogenes* = Ea, *Escherichia coli* = Ec, *Fusarium oxysporum* = Fo, *Klebsiella oxytoca* = Ko, *Klebsiella pneumoniae* = Kp, *Mycobacterium tuberculosis* = Mt, *Proteus mirabilis* = Pm, *Proteus vulgaris* = Pv, *Pseudomonas aeruginosa* = Pa, *Salmonella typhii* = Sti, *Salmonella typhimurium* = St, *Serratia marcescens* = Sm, *Staphylococcus aereus* = Sa, *Streptococcus pyrogens* = Sp.

from five plants growing within a 2 m^2 area, in the same strata and minimizing the impact of soil composition.

10.2.2 Extraction

10.2.2.1 Preparation of plant extracts

Fresh subterranean plant material was used in this study. One of the primary focus areas was to combine ample fresh material from five plants growing very close to each other; in order to collect viable samples from the freeze dried extracts. After collection the roots were cut into smaller pieces; approximately 1.0 cm^2. Approximately 121 g of these processed roots were added to 3.5 L of boiling tap water. At fixed time intervals (T_{00}, T_{05}, T_{10}, T_{15} and T_{20} minutes) 500 ml of the boiling liquid was removed from the pot. These volumes were separately filtered through Whatmann No. 1 filter paper, and the filtrate stored in appropriately labelled glass containers. The filtrates were frozen; where after they were dried via freeze drying (VIRTIS, United Scientific (PTY) Ltd.). The dried, concentrated extracts were used to prepare 50 mg/ml stock solutions to be used in the micro-dilution assays.

Time 0 (T_{00}) was considered the removal of a 500 ml volume within 15–20 seconds after adding the plant material to the boiling water. The other samples were collected at 5 minute intervals succeeding T_{00}.

10.2.3 Antimicrobial activity

10.2.3.1 Bacterial and fungal cultures

The choice of pathogens for investigation was based on STIs commonly associated with an abnormal urethral discharge. These were *N. gonorrhoeae* (ATCC 49226) and *Proteus vulgaris* (ATCC 6380) two Gram-negative bacteria, and *Candida albicans* (ATCC 10231) a Gram-positive fungus. *Staphylococcus aureus* (ATCC 25923), a

Gram-positive bacterium was also included in the test protocol. Where no reference strains were available clinical strains were used.

Neisseria gonorrhoeae was obtained from ATCC as KWIK-STIK® plus microorganism. The bacteria were inoculated onto chocolate agar (Oxoid GC agar base) which was supplemented with 2% (W/V) of haemoglobin and 1% (v/v) of Vitox supplement; where after it was incubated for 24 h at 37 ˚C in 5% CO_2. A number of colonies of pure culture were collected from an overnight culture and suspended in 5 ml Mueller-Hinton (MH) broth. The turbidity of this cell suspension, at 540 nm, was adjusted by adding either MH broth or organism as required, until the turbidity of the suspension was equivalent to that of a 0.5 McFarland $BaSO_4$ standard to approximately 108 CFU/ml.

Candida albicans, *P. vulgaris* and *S. aureus* were inoculated onto nutrient broth. With the exception of *C. albicans* (48 hr), the incubation period was 24 hr. The turbidity of all suspensions was adjusted using the McFarland standard.

10.2.3.2 Microdilution method

The minimum inhibitory concentration (MIC) values were determined using the micro-titre plate dilution method adapted from Eloff (1998). In general all assays were performed in triplicate; the only exception being some of the T_{00} samples, where the yield of active compounds was so small that only duplicate assessments could be performed. Stock solutions of the crude plant extracts were made up to 50 mg/ml using distilled water; dimethyl sulfoxide (DMSO) was not required as the concentrates were initially prepared from aqueous solutions. Nutrient broth (100 µl) was pipetted into all wells of the micro-titre plate. Thereafter, stock solutions (100 µl) of each of the prepared extracts were transferred into the first row (row A) of the micro-titre plate. Serial dilutions were performed, starting at 12.5 mg/ml and ending at 0.09 mg/ml. A standard volume of 100 µl of culture was added to all 96 wells. The micro-titre plates were then incubated at 37 °C for the appropriate period of time, as required by the specific pathogen. Following the period of incubation, 40 µl of 0.2 mg/ml p-iodonitrotetrazolium violet (INT, Sigma) was added to all wells. After

addition of INT the micro-titre plates were incubated for 30 minutes at 37 °C. The INT viability indicator was added to determine visually where microbial growth changed the colour of the solution; where a red-pink colour indicated viable bacteria and clear-yellow indicated inhibition. The inclusion of negative controls ensured that the relevant nutrient or MH broth was not contaminated. The broad spectrum antibiotic Ciprofloxacin was used as a positive control; serial diluted from 0.25 mg/100 µl.

10.3 RESULTS AND DISCUSSION

In this *in vitro* study, on the antimicrobial potential of aqueous extracts prepared from *C. roseus* roots, the only noteworthy activities (MIC ≤ 1 mg/ml) detected were those for *N. gonorrhoea* (Table 10.2). The MIC concentrations for the other pathogens ranged from 6.25 to 12.5 g/ml. No T_{10} antimicrobial assessments could be performed as this extract was lost when the container broke during the freezing process.

Herbal medicines are an invaluable primary health care resource; especially in rural settings where such medicines are often the only ones available. The progression from traditional medicine, through phytochemical screening, up to its use in allopathic medicine is well documented for many species; including but not limited to *C. roseus.*

This study reflected on the ethnobotanical use (Erasmus et al., 2012) and preferred preparation method of root extracts (Semenya, 2012) from *C. roseus*, among the Bapedi residing in the Limpopo Province of South Africa. Roots are the only plant part used by Bapedi traditional healers and it is exclusively applied in the treatment of gonorrhoea, commonly referred to as “drop” in the light of the diagnostic presence of an abnormal urethral discharge (Jones and Lopez, 2006). This perception might be somewhat premature and erroneous as a number of pathogens can cause this abnormal discharge. However, in the traditional medicinal practice accompanied by the absence of clinical confirmation regarding the identification of specific pathogens involved, the implication of pathogens such as *N. gonorrhoea*, *C.*

Table 10.2: Impact of boiling time on the bactericidal and antifungal efficacy of aqueous *Catharanthus roseus* root extracts.

Pathogens	Minimum inhibitory concentrations (mg/ml) of *Catharanthus roseus* root extracts			
	T_{00}	T_{05}	T_{15}	T_{20}
Neisseria gonorrhoea	**0.78**	**0.78**	**0.78**	**0.78**
Proteus vulgaris	12.50	6.25	12.50	12.50
Candida albicans	12.50	6.25	6.25	6.25
Staphyllococcus aureus	6.25	6.25	6.25	6.25
Ciprofloxacin	<0.09 mg/ml against all tested pathogens			

albicans and *P. vulgaris* cannot be excluded. In line with the described preparation method employed by the Bapedi (Erasmus et al., 2012; Semenya, 2012), the current study focussed on two aspects. Firstly the fact that only water was used as an extraction medium; and secondly, the disparity found in the reported preferred boiling times, which ranged from 5 to 20 minutes; hence our approach to collect extracts at 5 minute intervals.

To the best of our knowledge, this is the first study to assess the bactericidal effect of crude *C. roseus* root extracts on *N. gonorrhoea*. An earlier study (van Vuuren and Naidoo, 2010) assessed the efficacy of leaf extracts against various pathogens, including *N. gonorrhoea*. Poor inhibitory response were recorded for both dichloromethane:methanol (MIC 4.0 mg/ml) and water (MIC >16.0 mg/ml) in their study. In contrast to this, our study found significant inhibitory activity (MIC 0.781 mg/ml) against this pathogen. Factors contributing to this might be that crude root extracts prepared from fresh material are superior to dried aerial parts; and that boiling, as opposed to a 24 h maceration period, mobilizes more bioactive compounds. However, the observation that the MIC value was unaltered throughout the boiling period is of some concern. This phenomenon supports the possibility that

the boiling period was either too short to significantly alter the bioactive profile, thus sustaining the initial response, or that it was long enough and that the inhibitory activity was maintained by a combination of substances that varied as the boiling period increased. The use of high performance liquid chromatography to assess the composition of these crude extracts will shed more light on this.

The Gram-negative bacterium, *P. vulgaris* was also implicated as a causative agent in urinary tract infections (O'Hara et al., 2000b). Ibrahim et al. (2011) used whole plant extracts with DCM:methanol (1:1) as extraction medium, and found inhibitory activity in the concentration range of 10–100 mg/ml. They also observed a dose-dependent response, as was reflected in the increased inhibition zones associated with an increase in the concentration. Furthermore Rath et al. (2012), using the disc diffusion method, reported zero activity for both aqueous and ethanol leaf extracts. The present study, employing the micro-dilution method (Eloff, 1998), found that *C. roseus* crude extracts collected after various boiling periods exhibited similar inhibitory capabilities. The fact that there was a slightly improved bactericidal effect after 5 minutes of boiling, which disappeared with extended boiling, is currently difficult to explain. It is possible that a specific compound or group of compounds were mobilised after 5 minutes of boiling and was present in a high enough concentration to affect the activity. This effect disappeared after a further 10 minutes of boiling (T_{15}) suggesting the following: (i) the initial compound/s was still present but at lower concentration, thus incapable of sustaining the T_{05} effect, or (ii) that the initial compound/s were still present in either high enough or reduced concentrations, but that its effect might have been antagonised by another compound/s that was mobilised after the extra 10 minutes of boiling.

Staphylococcus areus, even though not strictly speaking causative in the etiology of STIs, have been implicated as an opportunistic infective agent in immune-compromised individuals (Buwa and Van Staden, 2006). Previous studies have reported on the efficacy of *C. roseus* extracts against this pathogen. Ramya et al. (2008) used the entire plant and extracted compounds with water, ethanol and methanol. With the exception of water, both ethanol and methanol extracts exhibited significant antibacterial activity. Ibrahim et al. (2011), using the entire plant, reported

a dose dependent inhibition with DCM:methanol extracts ranging in concentration from 10–100 mg/ml. In contrast to this, Govindasamy and Srinivasan (2012) collected plant parts separately. They used ethanol and methanol to extract compounds, and found that the ethanol extracts exhibited better antibacterial activity than those of the methanol extracts. Ethanolic root extracts exhibited the best antibacterial activity. In the present study antibacterial activity against *S. aureus* was consistent and independent of boiling time. It was, however, in contrast to that of Ramya et al. (2008) who reported no activity for aqueous root extracts against *S. aureus*. Our results clearly illustrate that boiling, as opposed to maceration at room temperature, is far more effective in extracting compounds.

The antifungal potential of *C. roseus* extracts is poorly investigated. The only scientific evidence that could be located was the study by Roy and Chatterjee (2010); that assessed fungicidal activities against *Fusarium oxysporum*, *Botrytis cinerea* and *Alternaria alternata*. No reference to the fungicidal activity of *C. roseus* against *Candida albicans* was found. The present study, even in the light of very poor antifungal activity is to the best of our knowledge the first assessment of this nature.

10.4 CONCLUSIONS

Noteworthy antimicrobial sensitivity towards the various aqueous extracts was only observed for *N. gonorrhoea*. Even though the inhibitory effect was sustained throughout the boiling period, the exact composition of the crude extracts needs to be affirmed in an effort to determine the primary contributing constituents. Findings in this study validate, in the absence of *C. albicans* infections, the traditional use of *C. roseus* root extracts to treat infections caused by *N. gonorrhoea* and identified via an abnormal urethral discharge. However, the Bapedi custom to prefer a boiling period of 20 minutes seems to be redundant as the inhibitory effect was present without any significant boiling.

SECTION 2
PHYTOCHEMICAL PROFILES: HIGH PERFORMANCE LIQIUD CHROMATOGRAPHY

Sample collection: action or process of collecting a small part or quantity intended to show what the whole is like

CHAPTER 11

Phytochemical analysis of *Aloe marlothii subsp. marlothii* crude aqueous extracts

Exploring *Aloe marlothii* subsp. *marlothii* aqueous extracts, in an effort to elucidate its antimicrobial efficacy: To what extent do boiling time influence activity levels and the phytochemical profile?

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ABSTRACT

The development of antimicrobial resistance has made the quest for alternative therapeutics, to control infectious diseases, imperative. Amongst these natural resources are several medicinal plants, such as *Aloe marlothii*, which is traditionally used in the treatment of sexually transmitted infections. The present study used previously reported antimicrobial activities to evaluate the effect of alternating boiling times; as observed among Bapedi traditional healers in the Limpopo Province, South Africa, on the phenolic, alkaloid and terpene profiles of *A. marlothii* extracts. Different parts of *A. marlothii* was extracted with water to yield crude extracts; which were subsequently subjected to HPLC-DAD analysis. No definite pattern for antimicrobial responses could be detected. *Neisseria gonorrhoea* was more susceptible to root and root/leaf extracts, than to leaf extracts. High Performance Liquid Chromatography results hint on the involvement of specific alkaloids and/or terpenes, rather than phenolics. The noteworthy response of *Proteus vulgaris* to only leaf and root extracts seems to correlate with terpene profiles, as there was no supporting evidence of phenolic and alkaloid involvement. The antifungal activity of *A. marlothii* aqueous extracts illustrated the sensitivity of *Candida albicans* to this species. The most promising compounds identified in the combined extract, but also present in the separate extracts, were an alkaloid (λ 254 nm RT = 21.392) and a

terpene (λ 220 nm RT = 17.253). Regarding *Staphylococcus aureus* the compound dynamics are complex and needs further investigation to assess possible synergistic and/or antagonistic interplays. The presented results lend credibility to the ethnobotanical use of these species in the treatment of sexually transmitted infections. It further supports the notion that boiling alters the phytochemical profile of extracts; sometimes for the better, improving antimicrobial activity, and occasionally for the worse.

Key words: Alkaloids, *Aloe marlothii*, Anti-microbial, Boiling time, Phenolics, Terpenoids.

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11.1 INTRODUCTION

Aloe marlothii A.Berger subsp. *marlothii* is a winter-flowering species that often occurs in extremely dense, impenetrable stands (Bredenkamp and Van Vuuren, 1987). This species often populate rocky north-facing slopes and mountainous regions, but can also be found in the grassland and savannah biomes of South Africa (Van Wyk and Smit, 2008). Similar to *Aloe ferox*, it is single stemmed and are protected from fire by persistent skirts of withered leaves around their stems. These "leafy skirts" around the stems of *A. marlothii* seems to be more prominent in fire prone habitats (Bond, 1983).

In the presence of far more prominent *Aloe* species, such as *A. vera* and *A. ferox*, which are commercially cultivated, it is not surprising that scientific studies validating the ethnobotanical therapeutics of this species is very limited. Phytochemical assessment, up to this point in time, focussed primarily on the presence of phenolics in leaf exudate (Van der Bank et al., 1995; Bisrat et al., 2000) and in root extracts (van Wyk et al., 1995). Plant phenolics represent a very large and diverse group of defensive compounds, and are defined as having a hydroxybenzene moiety. Amongst these compounds are some simple phenols noted

for their antimicrobial, antiseptic and topical antimicrobial properties; some can also inhibit prostaglandin synthetase and/or 5-lipoxygenase (Polya, 2003). The latter clearly supporting the contribution of phenols to anti-inflammatory responses.

In the modern laboratory set-up solvents with different polarities, such as acetone, hexane and ethanol are used to target the isolation of specific compounds from plant material. However, in comparison to this the traditional healer must resort to far more rudimentary procedures when preparing herbal remedies. Mostly, water is used as the extraction medium and the plant material; dried and powdered or fresh is then added to it. The preparation process of aqueous extracts varies, sometimes only slightly and at other times quite significantly, depending on the religious and cultural confines of the ethnic group. Such a discrepancy with regard to preferred boiling time was noted among Bapedi traditional healers, residing in the Limpopo Province of South Africa (Semenya, 2012), specifically but not limited to the therapeutic application of *A. marlothii*. The focus of the present chapter is therefore to assess the impact of various boiling periods on the phytochemical profile of *A. marlothii* leaf and root extracts, and to compare this to the previously reported remarkable antimicrobial responses.

11.2 METHODS AND MATERIALS

11.2.1 Sample collection

Plant materials were collected in February 2013, from Ga-Mamadila village, Capricorn District, Limpopo Province (S 23°47’17.8”; E 29°13’36.4”). Voucher specimens were collected and deposited at the Larry Leach Herbarium (UNIN) of the University of Limpopo. Duplicate leaf and root material was collected, during summer, from three plants growing in close proximity of each other; thus in the same soil strata.

11.2.2 Extraction

11.2.2.1 Preparation of plant extracts

In this study only fresh plant material was used. After collection the leaves and roots were cut into smaller pieces. Preparing the leaf extracts; 425 g of leaf material was added to 2.7 L of boiling tap water. Similarly, 426 g of root material was boiled for the preparation of the root extracts. During the preparation of the multi-part extract 232 g of leaf material was added to 235 g roots, which was boiled together. At specific time intervals (T_{00}, T_{05}, T_{10}, T_{15} and T_{20} minutes) 500 ml of boiling liquid was removed, and filtered through Whatmann No. 1 filter paper. No extra water was added (no topping up) in order to concentrate the extract so that the highest possible yield of bioactive compounds could be collected. Filtered extracts were frozen; where after the water was removed via freeze drying (VIRTIS, United Scientific (PTY) Ltd.). The dried, concentrated extracts were used to prepare 50 mg/ml stock solutions which were used in the micro-dilution assays.

Time 0 (T_{00}) was considered the removal of a 500 ml volume within 15–20 seconds after adding the plant material to the boiling water. The other samples were collected at 5 minute intervals succeeding T_{00}.

11.2.3 Quantitative analysis by High Peformance Liqiud Chromatography

High Peformance Liqiud Chromatography (HPLC) analyses were performed on a Shimadzu instrument (Shimatzu Corp., Kyota, Japan) equipped with a quaternary pump, an autosampler, column thermostat and a diode-array-detector (DAD) with a sampling frequency of 2 Hz. Chromatographic sample separation was carried out on a Discovery® Bio wide pore C_{18} column (4.0 mm i.d. x 25 cm) at 40 °C. Elution was performed with a flow rate of 1.0 ml/min. The binary mobile phase consisted of acetonitrile (A) and ammonium acetate, 10 mmoL (B). The following gradient elution was employed: 10% A (2 min), 10–30% B (10 min), 30–45% B (50 min), 70–80% B (65 min) and 80–95% B (70 min). Subsequent to the running, the gradient was set

back to 10% A and the system was allowed to reach equilibrium, where after an injection volume of 20 µl was used. Spectral data from all peaks were recorded over the 200–400 nm range, and integrated at 254 nm for alkaloids, 320 nm for phenolics, and 220 nm for terpenes.

11.3 RESULTS

The quantitative study of the terpene (λ = 220 nm), alkaloid (λ = 254 nm) and phenolic (λ = 320 nm) compounds present in *A. marlothii* aqueous extracts was performed by HPLC-DAD. In this study the focus was on boiling-time dependent peak patterns that followed a similar trend as that observed in the previously reported MICs (Chapter 8).

Table 11.1 summarises the number of peaks observed at each of the mentioned wavelengths; including the pathogens tested together with their reported MIC values. These data sets are categorised according to boiling time intervals (T_{00}-T_{20}). Tables 11.2 to 11.5 summarises the number of peaks, at each wavelength, that exhibited similar trends as those observed for the noteble MIC responses, for the various pathogens included in this test protocol.

Table 11.1, clearly illustrates that no definite pattern regarding the number of compound peaks is present. In most cases the boiling period resulted in an initial increased availability of some compounds, which either continued to increase in availability or decreased with time. All compound groups tested exhibited varied rates of compound availability as boiling time progressed.

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Table 11.1: Effect of boiling on the terpene, alkaloid and phenolic chromatogram peak profiles of crude extracts, prepared from *Aloe marlothii* leaves and roots.

Boiling time	Pathogen	Plant parts together with associated MIC (mg/ml) responses and wavelengths for compound detection											
		Leaves				Roots				Leaves and roots			
		MIC	220 nm	254 nm	320 nm	MIC	220 nm	254 nm	320 nm	MIC	220 nm	254 nm	320 nm
T_{00}	***Ng***	1.56	81	98	55	3.12	178	77	17	**<0.09**	164	57	19
	Pv	**0.78**				**0.39**				**0.39**			
	Ca	**0.39**				**0.78**				**0.78**			
	Sa	1.56				1.56				**0.19**			
T_{05}	***Ng***	1.56	91	124	91	3.12	191	124	40	**0.78**	163	110	41
	Pv	1.56				**0.39**				**0.39**			
	Ca	**0.78**				**0.78**				**0.78**			
	Sa	3.12				1.56				**0.78**			
T_{10}	***Ng***	6.25	90	138	81	1.56	185	112	40	**0.78**	168	124	59
	Pv	**0.78**				**0.39**				**0.78**			

	Ca	**0.78**				**0.78**				**0.78**			
	Sa	3.12				**0.78**				**0.78**			
T_{15}	***Ng***	6.25	186	131	60	**0.78**	182	119	48	**0.78**	164	136	60
	Pv	1.56				**0.78**				12.5			
	Ca	**0.78**				**0.39**				**0.78**			
	Sa	12.5				**0.78**				**0.78**			
T_{20}	***Ng***	6.25	178	145	56	**0.78**	184	137	59	**0.78**	94	80	50
	Pv	1.56				**0.78**				12.5			
	Ca	**0.78**				**0.39**				**0.78**			
	Sa	12.5				**0.78**				**0.39**			

Pathogen key: ***Ng*** = *Neisseria gonorrhoea*, ***Pv*** = s*Proteus vulgaris*, ***Ca*** = *Candida albicans* and ***Sa*** = *Staphylococcus aureus*. Noteworthy MIC values (< 1 mg/ml) are bold.

Wavelenghts: terpenes (λ = 220 nm), alkaloid (λ = 254 nm) and phenolics (λ = 320 nm).

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Table 11.2: Chromatogram peaks that mimic the MIC (mg/ml) pattern observed for *Neisseria gonorrhoea*, when treated with *Aloe marlothii subsp. marlothii* root and root/leaf aqueous extracts.

MIC (mg/ml)											
Amw**	3.12	3.12	1.56	**0.78#**	**0.781#**	**Ambw*****	**0.09#**	**0.78#**	**0.78#**	**0.78#**	**0.78#**
HPLC peaks (phenolics, λ = 320 nm)											
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**	**RT***	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**
22.731	0.000	0.000	0.000	0.430	0.452	**4.077**	0.479	0.216	0.186	0.213	0.062
						21.363	39.952	45.404	43.100	43.346	13.679
HPLC peaks (alkaloids, λ = 254 nm)											
6.168	0.000	0.000	0.000	0.043	0.039	**24.929**	0.101	0.056	0.052	0.045	0.043
12.946	5.142	3.947	3.813	3.210	2.985						
25.333	0.000	0.000	0.000	0.054	0.051						
34.492	0.000	0.000	0.000	0.090	0.082						
93.042	19.177	10.550	13.298	11.327	8.113						
HPLC peaks (terpenes, λ = 220 nm)											
2.338	0.038	0.702	0.058	4.159	3.914	**5.655**	0.003	0.948	1.227	1.009	0.773
17.395	0.247	0.098	0.274	0.344	0.322	**10.815**	0.009	0.103	0.132	0.218	0.195
35.534	0.065	0.052	0.061	0.122	0.132	**16.833**	0.352	0.201	0.133	0.168	0.136

36.184	0.011	0.011	0.008	0.021	0.017	**20.271**	0.011	0.025	0.019	0.028	0.033
36.429	0.000	0.000	0.000	0.032	0.025	**21.361**	2.149	3.729	4.117	5.201	4.050
39.747	0.040	0.130	0.139	0.191	0.217	**21.802**	0.010	0.020	0.004	0.028	0.025
40.075	0.000	0.000	0.000	0.007	0.007						
42.836	0.005	0.002	0.011	0.014	0.013						
58.577	0.000	0.000	0.000	0.008	0.012						
68.134	0.121	0.074	0.069	0.067	0.058						
69.833	0.000	0.000	0.000	0.006	0.004						
79.542	0.085	0.060	0.075	0.059	0.056						

*RT = retention time obtained from respective chromatograms; **Amw = *A. marlothii* roots and ***Ambw = the combination of *A. marlothii* leaves and roots.

[#]Noteworthy MIC (<1 mg/ml) in bold. Blank spaces = no further relevant data recorded.

Table 11.3: Chromatogram peaks that mimic the MIC (mg/ml) pattern observed for *Proteus vulgaris*, when treated with *Aloe marlothii* root, leaf and root/leaf aqueous extracts.

MIC (mg/ml)											
Amw**	**0.39**$^{\#\#}$	**0.39**$^{\#\#}$	**0.39**$^{\#\#}$	**0.78**$^{\#\#}$	**0.78**$^{\#\#}$	**Amb*****	**0.78**$^{\#\#}$	1.56	**0.78**$^{\#\#}$	1.56	1.56
HPLC peaks (phenolics, λ = 320 nm)											
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**	**RT***	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**
No relevant combinations detected						**93.745**	15.192	9.851	14.631	3.588	4.433
HPLC peaks (alkaloids, λ = 254 nm)											
4.501	0.245	0.390	0.495	0.265	0.213	**25.575**	0.824	0.598	0.777	---	0.515
12.952	5.142	3.047	3.913	3.210	2.885	**38.763**	0.776	0.199	0.640	0.377	0.182
14.261	3.410	------	2.946	1.345	1.589	**93.746**	7.149	5.433	9.122	3.862	3.449
15.772	0.509	0.594	0.585	0.461	0.478						
26.252	0.250	0.613	0.801	1.089	1.038						
42.796	----	0.125	0.125	0.153	0.168						
HPLC peaks (terpenes, λ = 220 nm)											
4.663	2.237	2.114	2.285	1.867	1.952	**21.305**	2.659	2.176	2.765	0.890	1.698
14.667	1.045	1.431	---	0.780	0.678						
19.844	0.033	0.030	0.035	0.024	0.023						

29.549	0.015	0.019	0.021	0.009	0.011
39.351	0.016	0.013	0.014	0.011	0.011
85.753	0.108	0.101	0.102	0.099	0.089
MIC (mg/ml)					
Ambw[#]	**0.39[##]**	**0.39[##]**	**0.78[##]**	12.5	12.5
HPLC peaks (phenolics, λ = 320 nm)					
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**
No relevant combinations detected					
HPLC peaks (alkaloids, λ = 254 nm)					
No relevant combinations detected					
HPLC peaks (terpenes, λ = 220 nm)					
3.938	1.680	2.422	3.279	3.733	----
11.463	0.019	0.017	0.006	0.004	0.004
14.341	1.178	1.085	0.823	0.779	0.543
24.491	0.019	0.019	0.016	0.008	0.001
35.754	0.095	0.089	0.039	0.024	----

*RT = retention time obtained from respective chromatograms; **Amw = *A. marlothii* roots, ***Amb = *A. marlothii* leaves and [#]Ambw = combined *A. marlothii* leaves and roots.

[##]Noteworthy MIC (<1 mg/ml) in bold. Blank spaces = no further relevant data recorded. ----- indicates that no data set was available for that specific boiling period.

Table 11.4: Chromatogram peaks that mimic the MIC (mg/ml) pattern observed for *Candida albicans*, when treated with *Aloe marlothii* root, leaf and root/leaf aqueous extracts.

MIC (mg/ml)											
Amw**	**0.78$^{\#\#}$**	**0.78$^{\#\#}$**	**0.78$^{\#\#}$**	**0.39$^{\#\#}$**	**0.39$^{\#\#}$**	**Amb*****	**0.39$^{\#\#}$**	**0.78$^{\#\#}$**	**0.78$^{\#\#}$**	**0.78$^{\#\#}$**	**0.78$^{\#\#}$**
HPLC peaks (phenolics, λ = 320 nm)											
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**	**RT***	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**
23.273	----	4.011	4.262	4.524	5.076	No relevant combinations detected					
24.041	----	2.596	2.095	3.255	3.388						
HPLC peaks (alkaloids, λ = 254 nm)											
3.059	----	0.705	0.727	0.866	1.012	**21.306**	29.766	46.326	43.888	40.250	44.641
3.288	----	2.257	2.286	2.802	3.118	**75.488**	0.149	0.086	0.079	0.075	0.076
3.663	----	0.411	0.451	0.506	0.694						
5.337	----	0.315	0.308	0.258	0.200						
10.841	----	0.722	0.606	0.911	1.058						
15.772	0.509	0.594	0.585	0.461	0.478						
25.587	----	0.093	0.096	0.125	0.137						
25.907	----	0.229	0.237	0.303	0.327						
26.252	----	0.613	0.801	1.089	1.038						

26.723	----	0.469	0.447	0.528	0.537						
39.757	----	0.246	0.282	0.345	0.384						
42.796	----	0.125	0.125	0.153	0.168						
HPLC peaks (terpenes, λ = 220 nm)											
4.663	2.237	2.114	2.285	1.867	1.952	No relevant combinations detected					
17.395	0.247	0.204	0.274	0.344	0.322						
19.844	0.033	0.030	0.035	0.024	0.023						
24.071	0.015	0.025	0.021	0.039	0.039						
32.736	0.011	----	0.009	0.017	0.019						
34.791	0.090	0.070	0.066	0.115	0.114						
35.140	0.060	0.072	0.055	0.141	0.159						
35.503	0.065	0.052	0.061	0.122	0.132						
MIC (mg/ml)											
Ambw[#]	**0.78[##]**	**0.78[##]**	**0.78[##]**	**0.78[##]**	**0.78[##]**						
HPLC peaks (phenolics, λ = 320 nm)											
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**						
No relevant combinations detected											
HPLC peaks (alkaloids, λ = 254 nm)											

21.392	41.119	52.659	54.428	48.001	23.966						
23.935	0.300	0.283	0.416	0.381	0.274						
HPLC peaks (terpenes, λ = 220 nm)											
3.574	1.744	1.534	1.386	1.528	1.072						
12.343	4.398	5.101	6.141	6.022	3.753						
13.432	2.495	1.616	1.802	2.493	1.872						
13.994	1.583	1.085	1.277	1.536	1.093						
14.777	0.795	0.659	0.660	0.779	0.543						
16.458	0.494	0.472	0.448	0.313	0.401						
17.253	0.240	0.258	0.221	0.266	0.217						
21.383	2.149	3.729	4.117	5.201	4.050						
24.491	0.019	0.019	0.016	0.008	0.010						
56.021	8.378	6.122	5.473	5.750	----						
68.113	0.077	0.068	0.060	0.071	----						
68.615	0.045	0.047	0.034	0.040	----						
79.513	0.077	0.060	0.066	0.057	----						
84.123	0.016	0.016	0.012	0.016	----						
93.055	1.179	0.846	0.778	0.633							

96.879	57.323	41.739	37.285	41.659							

*RT = retention time obtained from respective chromatograms; **Amw = *A. marlothii* roots, ***Amb = *A. marlothii* leaves and #Ambw = combined *A. marlothii* leaves and roots.

##Noteworthy MIC (<1 mg/ml) in bold. Blank spaces = no further relevant data recorded. ----- indicates that no data set was available for that specific boiling period.

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Table 11.5: Chromatogram peaks that mimics the MIC (mg/ml) pattern observed for *Staphylococcus aureus*, when treated with *Aloe marlothii* root and root/leaf aqueous extracts.

MIC (mg/ml)											
Amw**	1.56	1.56	**0.78#**	**0.78#**	**0.78#**	**Ambw*****	**0.19#**	**0.78#**	**0.78#**	**0.78#**	**0.39#**
HPLC peaks (phenolics, λ = 320 nm)											
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**	**RT***	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**
No relevant combinations observed						**45.448**	3.198	8.002	7.664	6.944	6.224
HPLC peaks (alkaloids, λ = 254 nm)											
3.808	----	0.387	0.539	0.504	0.516	**3.470**	0.094	0.646	0.783	0.662	0.391
55.993	17.696	----	9.393	8.182	6.076						
HPLC peaks (terpenes, λ = 220 nm)											
14.595	1.045	1.431	0.738	0.780	0.678	**3.135**	0.128	1.384	1.259	----	0.999
20.658	0.019	0.011	0.008	0.008	----	**3.330**	0.678	1.117	1.039	----	0.847
33.989	0.152	0.216	0.420	0.424	----	**3.941**	1.680	----	3.279	3.733	2.531

*RT = retention time obtained from respective chromatograms; **Amw = *A. marlothii* roots and ***Ambw = combined *A. marlothii* leaves and roots;

#Noteworthy MIC (<1 mg/ml) in bold. ----- indicates that no data set was available for that specific boiling period.

11.4 DISCUSSION

The tremendous chemical diversity of natural products (NP) has been a major contributor towards pharmaceutical discovery of novel drugs. Pathogenic resistance to known antimicrobials clearly indicate how crucial these NP-derived therapeutics are regarding their antimicrobial potential. However, despite advances in drug discovery, the growing number of immune-suppressed patients presents new challenges to health care systems, especially where co-infections are concerned.

Interest in traditional medicine, as a modality focussing primarily on the use of NPs, and the various approaches employed in the preparation of herbal remedies, has resulted in a plethora of scientific validations for the use of plant extracts as therapeutics (Bruschi et al., 2011; De Wet et al., 2012; Naidoo et al., 2013). Quite often these crude extracts are further scrutinised in an effort to identify and extract the active principle/s which may form important leads in future drug development. However, all of this starts further back, in the traditional healer's practice where years of experience and trial and error culminates in the identification of the best possible species, plant or plant part combinations and extraction procedures to treat specific ailments. It is unfortunate that modern science often underestimates the value of traditional preparation procedures, as modern science tend to focus more on the specific plants or parts used as well as their application in multi-plant or multi-plant part extracts. This reluctance to focus primarily on aqueous extraction might partially be supported by scientific evidence indicating the precarious antimicrobial responses associated with such extracts (Jäger, 2003; Motsei et al., 2003; Mohana et al., 2008; De Villiers et al., 2010). Yet, further investigations into the preparation of such aqueous extracts in modern laboratories would more often than not indicate that the procedure had nothing, or very little, in common with the traditional approach, except for the fact that water is used. It is therefore not surprising that some investigations regarding ethnobotanical medicinal usage unsuccessfully address the efficacy of aqueous extracts in *in vitro* studies. Nevertheless, the use of more refined extraction methods employing solvents such as acetone, hexane, methanol and dichloromethane cannot be discarded at the cost of aqueous

extraction. These other solvents, with their unique polarities, are frequently used to successfully extract specific compounds that do exhibit exceptional antimicrobial activity (De Villiers et al., 2010; Mahlo et al., 2013). It is acknowledged that the quest for the development of antimicrobials necessitate such steps; however, scientific validation of traditional remedies should first and foremost focus on if that specific plant, plant combination prepared via the said procedure can indeed do what it is perceived to do within the confines of traditional medicine.

The present study focussed primarily on the boiling period discrepancy that was reported in an earlier ethnobotanical report amongst the Bapedi (Semenya, 2012). Accordingly it was noted that boiling times, during extract preparation, varied from 5–20 minutes. Boiling time did indeed alter the bio-availability patterns for terpenes, alkaloids and phenolics (Table 11.1); these fluctuations are most probably related to the unique physico-chemical nature of these compounds, which will ultimately determine their rate of release and consequently either their increased or reduced availability as boiling progressed. At each respective wavelength that was used for detection of these compounds, peak patterns were identified, or sometimes not, that mimicked the MIC response of the pathogen tested.

Plant phenolics are quite complex and exhibit a diverse structural range, thereby providing some molecular rationale for their varied therapeutic impact (Daglia, 2011). Van der Bank et al. (1995) and Bisrat et al. (2000) confirmed the presence of phenolics in leaf exudate of *A. marlothii*. Phenolics, especially flavonoids, are well-known for their antimicrobial and anti-inflammatory potential (Harborne and Baxter, 1999). Earlier findings (Table 8.1) did not report any significant antimicrobial activities against *N. gonorrhoea*, when leaf extracts were used. However, employing aqueous root extracts, noteworthy inhibitory responses against *N. gonorrhoea* became apparent after 10 minutes of boiling (MIC = 0.781 mg/ml). Van Wyk et al. (1995) confirmed the presence of phenolics, such as chrysophanol, asphodelin, aloesaponarin I and aloesaponol I and II in the roots of *A. marlothii* might shed some light on the compounds involved in this response. It is highly unlikely, albeit not impossible, that this could be attributed to the single relevant peak (RT = 22.731) observed within the phenolic wavelength range. A far

more plausible explanation might be found within the larger number of peaks detected in the alkaloid and terpene ranges; both groups are known to contain compounds that possess antimicrobial activity. In the alkaloid range it is highly improbable that the two major peaks (RT = 12.946 and 93.042) contributed to the noteworthy MIC response closer to the end of the boiling period ($T_{15–20}$); especially if it is considered that their availability decreased dramatically. The remaining three peaks reflected on compounds present in minute concentrations; however, their increased availability towards the end of the boiling period supports the possibility that either one or more of these are potent enough to elicit this response or that a synergistic effect exist amongst them. Twelve peaks in the terpene range supports the MIC response; most of which were present in small amounts as reflected in a chromatogram area surface <1%. Synergism is possible amongst those compounds available in lesser amounts; though the striking increased concentration observed at RT = 2.338, seems to be a more appropriate candidate to support the observed antibacterial response.

Even though aqueous leaf extracts were relative ineffective against *N. gonorrhoea*, when leaves and roots were combined the inhibitory response against this pathogen were dramatically altered; with the lowest MIC (0,091 mg/ml) recorded at T_{00}. The MIC from T_{05} to T_{20} remained noteble but constant at 0.781 mg/ml, whereas the root only extracts showed no noteworthy response during intervals T_{00} to T_{15}. The reduced number of relevant peaks within all detected ranges (Table 11.2) makes an explanation quite challenging; however, the peak range (RT: 25.333 and 24.929) shows promise as the concentrations available supports the magnitude of the MIC response. This is further supported by the fact that no other peaks were found that appeared in both extracts and at concentrations relevant to the antimicrobial response. The possibility that an alkaloid, present in such small amounts, can induce such a remarkable inhibitory response warrants further investigation.

The use of aqueous *A. marlothii* root, leaf and combined root/leaf extracts to test the susceptibility of *P. vulgaris*, yielded limited but interesting results. Most prominently was the fact that both leaf and root extracts, used separately, performed

better than when combined; especially in the $T_{15–20}$ range where the MIC for the combined extract increased to 12.5 mg/ml (Table 11.3). The most plausible explanation for the loss in inhibitory potential observed might be attributed to the lack of phenolic and alkaloid compounds mimicking the MIC response. Regarding *P. vulgaris*, the presence of specific terpenes seems to play an important role in the inhibitory potential of the root extracts; which may in part also explain the inhibitory responses observed within the first 15 minutes with the combined extract.

Candida albicans is often implicated in reproductive health issues (McCullough et al., 1996; Jones and Lopez, 2006; Masoko et al., 2007; Mulaudzi et al., 2011; Jaggi et al., 2012). It does have the potential to cause an abnormal, smelly urethral discharge, and as such cannot be excluded from antimicrobial assessment; particularly when traditional healers use this discharge as one of their diagnostic criteria. It is evident that *C. albicans* is extremely susceptible to aqueous extracts, especially when plant extracts demonstrate the ability to inhibit *C. albicans* yeast-hyphal transition (Chevalier et al., 2012). Similar to this susceptibility to aqueous extracts, we recently confirmed (Table 8.1) that *C. albicans* exhibited remarkable sensitivity to extracts prepared from fresh *A. marlothii* root and leaf material. All inhibitory responses, irrespective of boiling time or the material used, was significant with MIC's < 1 mg/ml (Table 11.4). Root extracts contained so many peak combinations that mimicked the MIC response that any one, or combination of them, could be responsible for the antifungal responses observed. This is further complicated by the fact that only the root extracts yielded peak combinations in all three detected wavelengths (λ = 220, 254 and 320 nm). Regarding the antifungal potential of the leaf and combined extracts; both lacked relevant compound groups when compared to the root extracts. However, the combined extract contained two compounds, an alkaloid (λ 254 nm RT = 21.392) and a terpene (λ 220 nm RT = 17.253); the former also being present in leaf extracts and the latter in root extracts. It is reasonable to conclude that these two compounds might be responsible for the antifungal potential of these aqueous extracts as observed throughout the various boiling periods. Further support for this is the fact that terpenes, specifically

saponins, have been judged to exhibit bioactivity against *C. albicans* hyphae formation (Bader et al., 2000).

In the assessment of the antibacterial potential of an extract, it is often advised that both Gram-negative and Gram-positive pathogens be included in the test protocol. This study was no exception, and *S. aureus* (Table 11.5) was included to evaluate the inhibitory capacity of these aqueous extracts. It is well documented that the increased susceptibility of Gram-positive bacteria, such as *S. aureus*, to plant extracts or therapeutics can be attributed to differences in its cell membrane (Palombo and Semple, 2001; Garvey et al., 2011), notably when compared to the more fastidious *N. gonorrhoea* that often exhibits tremendous resistance against known antibiotics. Leaf and combined leaf/root extracts inhibited *S. aureus*; however, even though only a limited number of peaks were identified, the compound dynamics are complex and needs further investigation to assess possible synergistic and/or antagonistic interplays.

It can be concluded that, contrary to the previous perception (McGaw et al., 2000), aqueous extracts of *A. marlothii* do possess scientifically validated antibacterial activity. High performance liquid chromatography clearly illustrated that boiling altered the phytochemical profile of the aqueous extracts to such an extent that in some scenarios antimicrobial efficacy was improved and in others it was gradually reduced. The involvement of compound groups in the antimicrobial responses detected seems to be dependent on the specific organism being tested, and requires further scrutiny to identify the key principles, as well as the antimicrobial mechanisms involved.

CHAPTER 12

Phytochemical analysis of *Ziziphus mucronata* crude aqueous multi-plant extracts

Antimicrobial efficacy of *Ziziphus mucronata*, *Hypoxis hemerocallidea* and *Tribulus terrestris* via aqueous multi extracts: Using High Performance Liquid Chromatography to explore the impact of boiling time on the phytochemical profile

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ABSTRACT

In an age of increased antimicrobial resistance, natural plant products are the mainstay of *de novo* therapeutics and their application in alleviating the burden of infectious diseases. The present study, an extension of previously reported antimicrobial activities related to *Ziziphus mucronata*, *Hypoxis hemerocallidea* and *Tribulus terrestris*, employed HPLC-DAD to assess phytochemical profile alterations, induced via different boiling times. The aim was to link fluctuations in the availability of alkaloids (λ = 254 nm), phenolics (λ = 320 nm) and terpenoids (λ = 220 nm) to specific antimicrobial activities. It was found that increasing the boiling period did alter the presence and availability of these secondary metabolites. Further analysis investigating similarities regarding the previously reported minimum inhibitory concentrations (MICs) when compared to corresponding trends in metabolite profiles revealed the following. *Neisseria gonorrhoea* and *C. albicans* were particularly susceptible to alkaloids and terpenoids; whereas *S. aureus* exhibited particular sensitivity to terpenoids. *Proteus vulgaris* exhibited resistance to almost all of the extracts, except *H. hemerocallidea* (T_{00}). This response can most probably be attributed to phenolic accessibility. In conclusion, the therapeutic properties of some extracts support their use in antimicrobial development. However, the possibility exist

that some of them are not predominantly antimicrobials and might induce their effect via anti-inflammatory modalities.

Key words: Alkaloids, Boiling, *Candida albicans*, *Neisseria gonorrhoea*, Phenolics, *Proteus vulgaris*, *Staphylococcus aureus*, Terpenes, Traditional healers.

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12.1 INTRODUCTION

Humans have been using natural products with therapeutic properties for centuries, without any rational explanation for their therapeutic impact. The rapid development of organic chemistry and pharmacology, since the early 19th century, contributed significantly to the understanding of which active principles contribute to a given therapeutic response (Stary, 1996). In South Africa, with its acclaimed biological and ethnic differences, an overwhelming floristic diversity of approximately 25 000 plant species (Steenkamp and Smith, 2006) contributes considerably to the traditional use of plants to treat an assortment of ailments, including, but not limited to, those infections resulting from exposure to a plethora of pathogens. In South Africa, traditional healers, without any standardisation regarding preparation methods or the combination of plant parts or even different plant species, use species such as *Ziziphus mucronata* (Sparg et al., 2000; Waterman et al., 2010), *Hypoxis hemerocallidea* (Mills et al., 2005; Philander, 2011) and *Tribulus terrestris* (Mabogo, 1990; Semenya, 2012) to treat various human and animal ailments; including sexually transmitted diseases.

A current global concern is the increased prevalence of curable sexually transmitted infections (STIs). This is not only based on the reported approximate global infection rate of nearly 444 million cases (WHO, 2011b), but also on the clinical concern that multidrug resistant pathogens challenges the efficacy of existing antibiotics, constituting a severe threat to human and veterinary medicine alike

(Wright, 2010). This highlights the urgent need for the development of *de novo* therapeutics, as well as adjustments to existing antimicrobial strategies.

Natural plant products remain one of the best resources of new antimicrobial compounds as it contains various secondary metabolites such as phenolics, terpenoids and alkaloids (Cowan, 1999; Stasiuk and Kozubek, 2010). These bio-active compounds are often structurally complex and HPLC can provide excellent analytical precision as well as an increased sample loading capacity in detecting them. The present chapter is an extension of previous antimicrobial assessments related to crude aqueous extracts prepared from *Ziziphus mucronata*, *Hypoxis hemerocallidea* and *Tribulus terrestris* (Chapter 9). Its focus is primarily on alterations in the phytochemical profile induced by various boiling intervals as employed by Bapedi traditional healers in the preparation of their remedies, and how these changes influenced the antimicrobial efficacy of relevant aqueous extracts against pathogens associated with STIs.

12.2 METHODS AND MATERIALS

12.2.1 Sample collection

Plant materials were collected in February 2013, from Ga-Mamadila village (Figure 9.1), Capricorn District, Limpopo Province (S 23°47’10.0”; E 29°13’42.8”). Voucher specimens were collected and deposited at the Larry Leach Herbarium (UNIN) of the University of Limpopo. The roots of *Ziziphus mucronata* subsp. *mucronata* (n = 3) and *Tribulus terrestris* (n = 6), and the tubers of *Hypoxis hemerocallidea* (n = 7) were collected where the species grew in close proximity of each other. This was done to ensure that samples were collected from species growing in the same strata, thus minimizing the effect of soil composition on the phytochemical profile.

12.2.2 Extraction

12.2.2.1 Preparation of plant extracts

Only fresh subterranean plant material was used in this study. The yield for all of these species were very small, thus it was decided to combine the fresh material collected from plants growing in close proximity of each other. After collection the roots and corms were cut into smaller pieces; to increase the release of bioactive compounds. The following weights of fresh root/tuber material was added separately to boiling tap water in a pot; 240 g of *Z. mucronata*, 850 g of *H. hemerocallidea*, 14 g of *T. terrestris*, and 100 g and 114 g, respectively, for the *Z. muconata* / *H. hemerocallidea* combination. The disparity in the *Z. mucronata* (57.4 g) and *T. terrestris* (12 g) combination relates to the fact that *T. terrestris* had a very low yield because only a small number of plants were available in the collection area, thus limiting the number of subterranean parts available. At specific time intervals (T_{00}, T_{05}, T_{10}, T_{15} and T_{20} minutes) 500 ml of the boiling liquid was removed from the pot. These extracted volumes were separately filtered through Whatmann No. 1 filter paper, and stored in appropriately labelled clear glass containers. Filtered extracts were frozen; where after the water was removed via freeze drying (VIRTIS, United Scientific (PTY) Ltd.). The dried, concentrated extracts were used to prepare 50 mg/ml stock solutions to be used in the micro-dilution assays.

Time 0 (T_{00}) was considered the removal of a 500 ml volume within 15–20 seconds after adding the plant material to the boiling water. The other samples were collected at 5 minute intervals succeeding T_{00}. No additional water was added (no topping up) in order to concentrate the extract so that the highest possible yield of bioactive compounds could be collected.

12.2.3 Quantitative analysis by High Peformance Liqiud Chromatography

HPLC analyses were performed on a Shimadzu instrument (Shimatzu Corp., Kyota, Japan) equipped with a quaternary pump, an autosampler, column thermostat and a

diode-array-detector (DAD) with a sampling frequency of 2 Hz. Chromatographic sample separation was carried out on a Discovery® Bio wide pore C_{18} column (4.0 mm i.d. x 25 cm) at 40 °C. Elution was performed with a flow rate of 1.0 ml/min. The binary mobile phase consisted of acetonitrile (A) and ammonium acetate, 10 mmoL (B). The following gradient elution was employed: 10% A (2 min), 10–30% B (10 min), 30–45% B (50 min), 70–80% B (65 min) and 80–95% B (70 min). Subsequent to the running, the gradient was set back to 10% A and the system was allowed to reach equilibrium, where after an injection volume of 20 µl was used. Spectral data from all peaks were recorded over the 200–400 nm range, and integrated at 254 nm for alkaloids, 320 nm for phenolics, and 220 nm for terpenes.

12.3 RESULTS

Tables 12.1 (*Z. mucronata* subsp. *mucronata* and *H. hemerocallidea*) and 12.2 (*Z. mucronata* subsp. *mucronata* and *T. terrestris*) outlines the number of peaks observed at each of the mentioned wavelengths; including the pathogens tested together with their respective MIC values. These data sets are categorised according to boiling time intervals ($T_{00–20}$).

Table 12.3, with its focus on the *Z. mucronata* subsp. *mucronata* and *T. terrestris* combination, outline the number of peaks, at each wavelength, that exhibited trends similar to those observed for the notable MIC responses against *Neisseria gonorrhoea*. In a similar way Table 12.4 reviews the data for the *Z. mucronata* subsp. *mucronata* and *H. hemerocallidea* combination against *C. albicans*. Table 12.5 sum up the various *Z. mucronata* subsp. *mucronata* multi-extracts that proofed themselves effective against *Staphylococcus aureus*.

Tables 12.1 and 12.2, illustrates that no definite pattern regarding the number of compound peaks could be found. In most cases the boiling period resulted in an initial increased availability of some compound groups, which either continued to increase in availability or decreased with time. Even in situations where the number of peaks remained unchanged from one boiling period to the next (Table 12.1 *Z.*

mucronata subsp. *mucronata* $T_{15\text{-}20}$ and Table 12.2 *T. terrestris* $T_{10–15}$), the chromatograms (data not shown) showed that the peak profiles were different.

CHAPTER 12

Phytochemical analysis of *Ziziphus mucronata* crude aqueous multi-plant extracts

Table 12.1: Effect of various boiling intervals on the phytochemical profile of crude extracts prepared from *Ziziphus mucronata* and *Hypoxis hemerocallidea*.

Time interval	Pathogens	*Ziziphus mucronata*				*Hypoxis hemerocallidea*				*Ziziphus mucronata* and *Hypoxis hemerocallidea*			
		MIC mg/ml	*220 nm	**254 nm	#320 nm	MIC mg/ml	*220 nm	**254 nm	#320 nm	MIC mg/ml	*220 nm	**254 nm	#320 nm
T_{00}	Ng	1.56	205	61	17	1.56	202	84	16	1.56	187	65	12
	Pv	1.56				**0.78**##				12.5			
	Ca	----				3.12				**0.19**			
	Sa	1.56				**0.78**##				**0.78**##			
T_{05}	Ng	3.12	202	101	26	3.12	193	148	62	1.56	199	114	27
	Pv	3.12				1.56				12.5			
	Ca	6.25				1.56				**0.19**##			
	Sa	1.56				1.56				**0.78**##			
T_{10}	Ng	1.56	198	90	28	6.25	199	153	59	1.56	207	101	23
	Pv	1.56				1.56				3.12			
	Ca	**0.39**##				1.56				**0.19**##			

	Sa	1.56				1.56				1.56			
T_{15}	Ng	1.56	211	94	24	6.25	192	154	60	1.56	205	108	26
	Pv	1.56				1.56				3.12			
	Ca	**0.19**$^{\#\#}$				6.25				**0.39**$^{\#\#}$			
	Sa	1.56				1.56				1.56			
T_{20}	Ng	1.56	209	102	24	12.5	197	153	47	1.56	218	158	41
	Pv	1.56				3.12				6.25			
	Ca	>12.5				3.12				12.5			
	Sa	1.56				3.12				1.56			

Pathogen key: ***Ng*** = *Neisseria gonorrhoea*, ***Pv*** = *Proteus vulgaris*, ***Ca*** = *Candida albicans* and ***Sa*** = *Staphylococcus aureus*. MIC in bold = noteworthy activity (<1 mg/ml). Wavelengths: terpenes*, alkaloids** and phenolics$^{\#}$. Significant antimicrobial responses $^{\#\#}$.

CHAPTER 12

Phytochemical analysis of *Ziziphus mucronata* crude aqueous multi-plant extracts

Table 12.2: Effect of various boiling intervals on the phytochemical profile of crude extracts prepared from *Ziziphus mucronata* and *Tribulus terrestris*.

Time interval	Pathogens	*Ziziphus mucronata*				*Tribulus terrestris*				*Ziziphus mucronata* and *Tribulus terrestris*			
		MIC mg/ml	*220 nm	**254 nm	#320 nm	MIC mg/ml	*220 nm	**254 nm	#320 nm	MIC mg/ml	*220 nm	**254 nm	#320 nm
T_{00}	Ng	1.56	205	61	17	6.25	94	99	89	3.12	183	119	38
	Pv	1.56				>12.5				12.5			
	Ca	----				>12.5				----			
	Sa	1.56				12.5				>12.5			
T_{05}	Ng	3.12	202	101	26	6.25	94	115	64	6.25	197	127	44
	Pv	3.12				>12.5				1.56			
	Ca	6.25				>12.5				**0.39**##			
	Sa	1.56				12.5				3.12			
T_{10}	Ng	1.56	198	90	28	6.25	86	105	66	**0.78**##	186	138	49
	Pv	1.56				>12.5				1.56			
	Ca	**0.39**##				>12.5				**0.78**##			

	Sa	1.56				12.5				1.56			
T_{15}	Ng	1.56	211	94	24	6.25	95	115	66	1.56	178	154	66
	Pv	1.56				>12.5				1.56			
	Ca	**0.19**[##]				12.5				**0.39**[##]			
	Sa	1.56				12.5				3.12			
T_{20}	Ng	1.56	209	102	24	6.25	121	130	85	3.12	169	176	90
	Pv	1.56				>12.5				1.56			
	Ca	>12.5				12.5				6.25			
	Sa	1.56				12.5				1.56			

Pathogen key: ***Ng*** = *Neisseria gonorrhoea*, ***Pv*** = *Proteus vulgaris*, ***Ca*** = *Candida albicans* and ***Sa*** = *Staphylococcus aureus*. MIC in bold = noteworthy activity (<1 mg/ml). Wavelengths: terpenes*, alkaloids** and phenolics[#]. Significant antimicrobial responses [##]. ----- indicates that no data set was available for that specific pathogen.

Table 12.3: Antimicrobial activity of the *Ziziphus mucronata* / *Tribulus terrestris* combination against *Neisseria gonorrhoea*, in conjunction with high performance liquid chromatography analysis of peak patterns mimicking the MIC response.

MIC (mg/ml)	3.12	6.25	**0.78**[##]	1.56	3.12
HPLC peaks (phenolics, λ = 320 nm)					
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**
**					
HPLC peaks (alkaloids, λ = 254 nm)					
2.624	1.768	2.502	1.171	1.324	0.754
4.084	0.000	0.840	4.178	2.500	4.417
13.032	0.000	2.854	1.497	2.230	0.926
22.776	0.255	0.258	0.134	0.202	0.511
54.193	4.041	4.765	3.105	4.308	2.020
55.895	16.683	15.243	12.414	14.097	6.333
56.281	7.491	6.745	5.437	6.328	2.812
62.219	0.000	0.088	0.101	0.069	0.063
HPLC peaks (terpenes, λ = 220 nm)					
1.397	0.445	0.054	**0.025**	0.044	0.327
6.612	0.625	0.924	**0.530**	1.006	1.437
12.857	0.000	2.289	**2.238**	2.747	4.373
18.059	0.100	0.575	**0.598**	0.526	0.382
19.790	0.009	0.416	**0.598**	0.525	0.173
21.799	0.026	0.896	**1.272**	1.045	0.629
22.827	0.018	0.587	**1.310**	1.167	0.264
28.310	0.013	0.327	**0.585**	0.364	0.009
29.364	0.010	0.310	**0.550**	0.328	0.013
30.414	0.005	0.310	**0.344**	0.241	0.006
32.711	0.017	0.137	**0.309**	0.257	0.006

33.958	0.318	0.967	**1.188**	0.695	0.201
34.562	0.074	0.346	**0.237**	0.571	0.534
36.788	0.007	0.569	**0.625**	0.497	0.016
38.721	0.000	0.509	**0.612**	0.405	0.008
39.558	0.017	0.145	**0.831**	0.663	0.001
47.758	0.003	0.323	**0.853**	0.332	0.001
52.288	0.013	0.077	**0.209**	0.112	0.015
52.996	0.077	0.226	**0.578**	0.143	0.001

*RT = retention time obtained from respective chromatograms. ** Indicates the specific wavelength where no combinations could be found that mimicked the observed MIC pattern. Significant antimicrobial activity[##].

Table 12.4: High performance liquid chromatography analysis of compound patterns resembling the MIC responses of *Candida albicans* when exposed to *Ziziphus mucronata* subsp. *mucronata* / *Hypoxis hemerocallidea* aqueous multi extracts.

MIC (mg/ml)	**0.78**[##]	**0.78**[##]	1.56	1.56	1.56
HPLC peaks (phenolics, λ = 320 nm)					
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**
**					
HPLC peaks (alkaloids, λ = 254 nm)					
**					
HPLC peaks (terpenes, λ = 220 nm)					
19.712	0.044	0.041	0.023	0.023	0.036
23.454	0.015	0.015	----	0.011	0.011
23.849	0.022	0.023	0.015	0.016	----
26.860	0.022	0.019	0.016	----	0.011
28.216	0.061	0.063	0.024	0.026	----
29.453	0.031	0.034	----	0.017	0.014

*RT = retention time obtained from respective chromatograms.** Indicates the specific wavelength where no combinations could be found that mimicked the observed MIC pattern. Significant antimicrobial activity[##]. ----- indicates that no data set was available for that specific boiling period.

Table 12.5: The impact of boiling time on the bioactive profile of *Ziziphus mucronata* multi extracts; and their accompanying antimicrobial activity against *Staphylococcus aureus*.

MIC (mg/ml)											
Zm	----	1.56	**0.39**$^{\#\#}$	**0.19**$^{\#\#}$	>12.5	**ZmHh**	**0.19**$^{\#\#}$	**0.19**$^{\#\#}$	**0.19**$^{\#\#}$	**0.39**$^{\#\#}$	12.5
HPLC peaks (phenolics, λ = 320 nm)											
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**	**RT***	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**
**						**					
HPLC peaks (alkaloids, λ = 254 nm)											
15.126	----	1.161	0.996	0.471	1.363	**					
HPLC peaks (terpenes, λ = 220 nm)											
2.076	----	0.529	0.516	0.296	0.827	**4.501**	1.313	1.160	1.140	1.061	0.762
44.203	----	0.075	0.059	0.030	0.279	**13.835**	1.623	1.631	1.659	1.574	1.133
						29.453	0.031	0.034	0.035	0.021	0.010
						56.121	6.735	6.263	6.720	6.919	14.400
						68.739	0.058	0.056	0.056	0.063	0.110
MIC (mg/ml)											
ZmTt	----	**0.39**$^{\#\#}$	**0.78**$^{\#\#}$	**0.39**$^{\#\#}$	6.25						
HPLC peaks (phenolics, λ = 320 nm)											

RT*	T_{00}	T_{05}	T_{10}	T_{15}	T_{20}						
**											
HPLC peaks (alkaloids, λ = 254 nm)											
12.544	----	2.232	1.847	2.165	1.362						
43.074	----	0.440	0.398	0.554	0.326						
HPLC peaks (terpenes, λ = 220 nm)											
4.605	----	0.435	0.526	0.481	1.180						
15.855	----	1.292	0.687	1.252	0.323						
16.302	----	1.104	0.942	1.189	0.268						
17.903	-----	0.762	0.598	0.747	0.151						
33.393	----	0.870	0.669	0.900	0.225						

*RT = retention time obtained from respective chromatograms. ** Indicates the specific wavelength where no combinations could be found that mimicked the observed MIC pattern. **Zm** = *Z. mucronata,* **ZmHh** = combined extracts of *Z. mucronata* and *H. hemerocallidea* and **ZmTt** = combined extracts of *Z. mucronata* and *T. terrestris*. Significant antimicrobial activity[##]. ----- indicates that no data set was available for that specific boiling period.

12.4 DISCUSSION

The therapeutic application of multi plant extracts, within the confines of traditional medicine, is often perceived as being more potent than single plant extracts. This phenomenon is well supported by the fact that diseases presenting with multiple symptoms are often associated with an increased health risk, hence the increased number of plant species used in the remedy and the more aggressive treatment approach.

Pathogens infecting the human reproductive system can adversely influence reproductive health in the old and the young. They have been known to affect prenatal development, frequently terminating in spontaneous abortions (Jones and Lopez, 2006), contribute towards maternal and neonatal sepsis (Seale et al., 2009), and to cause urinary tract infections in the elderly (Matthews and Lancaster, 2011). The most concerning aspect is that these, predominantly, sexually transmissible pathogens regularly present asymptomatically with no symptoms present to assist in its diagnosis. Clearly, within modern medicine this is not really a concern as screening for pathogens and possible co-infections are done regularly when behavioural traits indicate an increased risk for STIs. On the other hand, traditional medicine faces many challenges in this regard as it relies mostly on symptoms, and asymptomatic individuals do not necessarily consult. This can easily, and erroneously, be perceived as a low incidence of STIs in a specific area, even though asymptomatic individuals do have the ability to infect their various partners.

When symptoms do present, traditional healers tend to focus on an abnormal urethral discharge (Erasmus et al., 2012; Semenya, 2012). The efficacy of a specific herbal remedy correlates with its ability to resolve this abnormal smelly urethral discharge. However, the etiology of such a discharge is quite diverse and often very complex in nature; and establishing antimicrobial activity for a crude extract (or the absence thereof) might only present part of its detected biological and/or physiological impact.

The focus of this study was to investigate a possible phytochemical rationale for the antimicrobial activity observed for aqueous multi-extracts of *Z. mucronata* subsp.

mucronata in combination with various other plant species. *Neisseria gonorrhoea*, *P. vulgaris*, *C. albicans* and *S. aureus* were exposed to single and combined aqueous extracts prepared via boiling times ranging from 5 to 20 minutes. The first aspect of interest was to use HPLC-DAD to establish whether fluctuations in the number of peaks detected at a specific wavelength supported the MIC responses (Tables 9.3 and 9.4). It became evident, very early on, that the answer/s were not to be found in mere numbers, and that no discernable patterns existed to support our antimicrobial findings.

The design of the present study, using the same plant material throughout the specific boiling period, enabled us to explore peaks that presented with a pattern similar to that of the noteworthy antimicrobial MIC when they were exposed to the various crude extracts. As an example, the MIC response of *N. gonorrhoea* to the combination of *Z. mucronata* subsp. *mucronata* and *T. terrestris* will be used to clarify the process. The only noteworthy response (MIC<1 mg/ml) was with the T_{10} extract. This pathogen became less susceptible from T_{00} to T_{05}, exhibited noteworthy susceptibility at T_{10} and gradually became less susceptible towards T_{20}. Applying this response pattern to the peak levels (area%) detected at each wavelength, the following was observed; no phenolics (λ = 320 nm) responded in a similar fashion; however, eight peaks within the alkaloid range (λ = 254 nm) and 19 peaks in the terpene range (λ = 220 nm) showed similar trends as those observed for the MICs. This line of reasoning was used throughout, to identify peak patterns where noteworthy activity was reported.

It is interesting to note that both *Z. mucronata* subsp. *mucronata* and *T. terrestris* exhibited some activity against *N. gonorrhoea,* even though not significant (MIC < 1 mg/ml). However, combining them did result in a notably improved inhibitory activity (MIC 0.78 mg/ml) after 10 minutes of boiling; an effect that was gradually lost as the boiling time progressed. Current findings suggest that phenolics were not involved in the response, but rather compounds (or any combination of) in the terpene and alkaloid ranges. *Ziziphus mucronata* subsp. *mucronata* is known for its microbicidal use and antimicrobial compounds in leaf extracts (Moloto, 2004); however, no studies reported on the antimicrobial assessment of root extracts.

Similarly *T. terrestris* leaf extracts did exhibit low antibacterial activity against various pathogens, except *N. gonorrhoea* (Mohana et al., 2008); still, antibacterial studies on root extracts against *N. gonorrhoea* is lacking. The current study is to the best of our knowledge, the first to describe this unique plant combination, its activity against *N. gonorrhoea* and the fact that this effect is most probably the result of the interaction between terpenes and alkaloids.

Proteus vulgaris, a Gram-negative bacterium, is often implicated in infections of the urogenital tract. None of the *Z. mucronata* subsp. *mucronata* combinations exhibited noteworthy activity against it. This observation was unexpected as *T. terrestris* was previously confirmed to possess inhibitory activity against *P. vulgaris* (Hussain et al., 2009); a response that was anticipated in the current study, but not found. The disparity between Hussain et al. (2009) and our study can probably be attributed to a number of reasons that include the fact that Hussain et al. (2009) are vague as to the precise plant part used, making it very difficult to say with certainty if similar plant parts were used. Furthermore their solvents did not include water as is the case in our study. However, *H. hemerocallidea*, in contrast to the existing literature, which lacks evidence supporting its antibacterial efficacy, did exhibit noteworthy inhibition of *P. vulgaris*. The current finding suggests that this inhibitory response might have been elicited via the impact of phenolic compounds. The antibacterial activity of these compounds is most probably exerted via its ability to act as a non-ionic surface active agent, which can disrupt the lipid-protein interface, or by protein denaturation and enzyme inactivation. Furthermore, phenols have the ability to damage membranes and alter its permeability, which could result in the uncoupling of oxidative phosphorylation, loss of metabolites as well as the inhibition of active transport (Boudet, 2007).

The yeast, *C. albicans*, showed very low susceptibility to *Z. mucronata* subsp. *mucronata* or to *T. terrestris*; including extracts prepared after combining these plants. This is supported by Usman et al. (2007) who reported a low MIC (6.25 mg/ml) for methanolic leaf extracts prepared from *T. terrestris*; highlighting the fact that using solvents with different polarities did not significantly alter the antifungal activity of the extracts. *Ziziphus mucronata* and its combination with *H.*

hemerocallidea exhibited promising antifungal activity. This activity was present at the onset of boiling (T_{00}) and disappeared towards T_{10}. These extracts illustrated the complexity of compound interactions and their concomitant antifungal activity. No relevant alkaloid or phenolic combinations were recorded; however, a number of peaks in the terpenoid range were identified. There is no doubt that terpenes have antimicrobial potential, as they have been reported (Polya, 2003) to contain compounds possessing antifungal activity. Even though the biological mechanisms involved in the antifungal activity of terpenoids are not fully understood, the following have been reported; the alteration of the mevalonate pathway (Mo and Elson, 2004), as well as destabilization of membranes affecting functions such as permeability and cell signalling, which leads to cell death (Brehm-Stecher and Johnson, 2003; Trombetta et al., 2005).

The Gram-positive bacterium *S. aureus* exhibited a diverse range of susceptibilities when exposed to the various aqueous extracts used in this study (Tables 9.3 and 9.4). Prevailing results propose that the antimicrobial efficacy of *Z. mucronata* combined with *H. hemerocallidea* and *T. terrestris* correspond well with the presence of terpenes. This is not surprising as many non-glycoside iridoids, sesquiterpenes and sesquiterpene lactones are known antimicrobials (Polya, 2003). Along similar lines the use of *H. hemerocallidea* in combination with *Z. mucronata*, involves the already confirmed activity of rooperol (a derivative of hypoxoside) against *S. aureus* (Laporta et al., 2007); also part of the plant terpenes.

In conclusion, the present HPLC-DAD data strongly suggests that boiling can alter the phytochemical profile of crude extracts. The study design identified terpenoids as the most prominent and promising antimicrobial group with inhibitory activity against *N. gonorrhoea*, *C. albicans* and *S. aureus*. This was followed by alkaloids with their inhibitory potential on *N. gonorrhoea* and *C. albicans*. Contrary to its well-known antimicrobial potential, phenolics were only identified as possible inhibitory agent for *S. aureus*. However, the limited antimicrobial contribution of phenolics in this study, and the poor antimicrobial responses observed for some of the aqueous extracts, argues in favour of yet another relevant mechanism. The presence of an abnormal urethral discharge can also be indicative of an

inflammatory response, thus if an extract exhibits no or very poor antimicrobial activity, but its use resolves this discharge it is fair to consider an anti-inflammatory contribution. The investigation into the anti-inflammatory capacity of the various aqueous extracts was not part of this study; and thus warrants further investigation to establish its contribution towards alleviation of the abnormal urethral discharge.

CHAPTER 13

Phytochemical analysis of *Catharanthus roseus* crude aqueous extracts

Catharanthus roseus, extraordinary Bapedi antigonococcal remedy: Phytochemical profile of aqueous root extracts subjected to variable boiling periods

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ABSTRACT

Catharanthus roseus is a renowned medicinal plant, appraised in both traditional and modern medicine. Traditionally all parts of this species are utilised, in crude extract preparation, to treat a plethora of ailments including but not limited sexually transmitted diseases. Preparation procedures differ significantly, as determined by ethno-religious concepts. The current study focussed on the impact of different boiling intervals on the phytochemical profiles of crude *C. roseus* root extracts, which were previously reported to exhibit noteworthy antogonococcal activity. Aqueous extracts were sampled, in 5 minute intervals, for a total boiling period of 20 minutes. High performance liquid chromatography was used to assess the phenolic (λ = 320 nm), alkaloid (λ = 254 nm) and terpenoid (λ = 220 nm) levels for each boiling period, and pair them with the previously reported antimicrobial responses. Chromatogram results clearly indicated an increased availability in the number of compounds as boiling time progressed. However, only a limited number of these compounds were available throughout the boiling period, and in approximately constant levels that mimicked the antimicrobial response. This study clearly indicated that alkaloids, more specifically monoterpene indole alkaloids, play a pivotal role in the antigonococcal activity of *C. roseus*. Feature research prospects should focus on

establishing the identity of these alkaloids as well as the possible synergistic or antagonistic interaction amongst them.

Key words: Aqueous extracts, Bapedi, Boiling, *Catharanthus roseus*, *Neisseria gonorrhoea*,

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13.1 INTRODUCTION

Catharanthus roseus (Apocynaceae), better known as the Madagascar periwinkle, has an impressive therapeutic record. Modern medicinal interest in this species was initially prompted by its indigenous therapeutic application as a hypoglaecemic agent, in the treatment of diabetes mellitus (Gurib-Fakim, 2006). However, it is agreed that it's most significant contribution to modern medicine was the discovery of terpenoid indole alkaloids, such as vinblastine and vincristine as well as their semi-synthetic clinically relevant derivatives (Mangeney et al., 1979; Hill, 2001), which dramatically and unequivocally changed cancer treatment protocols (van der Heijden et al., 2004). Since then many studies assessed its therapeutic potential as hypoglycaemic (Van de Venter et al., 2008; Patel et al., 2012; Yao et al., 2013), antihyperlipidemic (Chauhan et al., 2012) and antimicrobial (Patil and Ghosh, 2010; Ibrahim et al., 2011; Kamaraj et al., 2012) agents.

The discovery of antimicrobial agents is unquestionably one of the 20th centuries' most prominent and cherished achievements. However, non-compliance to the correct and effective use of existing antimicrobials resulted in the emergence of microbial resistance. This increased the negative impact on health care services; especially in large developing countries (Okeke et al., 2005). The advent of pathogen resistance has impeded the rate at which new antimicrobials are introduced into the public domain (Russell, 2002); and many initiatives have been started to exploit natural plant products as sources of innovative therapeutic agents (Clardy and

Walsh, 2004). Since phytochemicals are more specific, bio-degradable and supposedly exhibit fewer side effects, they present an unequalled platform for structural diversity and biological functionality (Nisbet and Moore, 1997; Verpoorte, 1998).

In South Africa a compelling floristic diversity of approximately 25 000 plant species (Steenkamp and Smith, 2006) contributes considerably to the traditional use of plants in the treatment of various ailments, including but not limited to those infections resulting from exposure to a plethora of sexually transmitted pathogens. In the light of such a floristic diversity the focus on ethnobotanical studies to identify specific species and plant parts used in the treatment of a particular disease provides a framework for phytochemical screening and pharmaceutical analysis. This approach was employed with the identification of *C. roseus* to treat “drop” (gonorrhoea), among the Bapedi in the Limpopo Province, South Africa (Erasmus et al., 2012; Semenya et al., 2012). These studies emphasised the fact that *C. roseus* aqueous root extracts, which was prepared via specific boiling periods, was exclusively used to treat gonorrhoea. No other medicinal application regarding this species has been reported among the Bapedi. Earlier antimicrobial assessment of aqueous root extracts confirmed noteworthy antimicrobial activity against *Neisseria gonorrhoea* (Table 10.2), thus validating its use by the Bapedi in the treatment of gonorrhoea. The focus of the present chapter is to use high performance liquid chromatography (HPLC) to evaluate the impact of various boiling intervals on the phytochemical profiles, and to relate this to the reported noteworthy antimicrobial activity.

13.2 METHODS AND MATERIALS

13.2.1 Plant collection

Plant materials were collected in February 2013, from Ga-Mamadila village, Capricorn District, Limpopo Province (S 23°47’15.2”; E 29°13’32.3”). Voucher specimens (Cr1, 2, 3, 4 and 5) were collected and deposited at the Larry Leach

Herbarium (UNIN) of the University of Limpopo. Subterranean parts were collected from five plants growing within a 2 m^2 area, in the same strata and minimizing the impact of soil composition.

13.2.2 Extraction

13.2.2.1 Preparation of plant extracts

Fresh subterranean plant material was used in this study. One of the primary focus areas was to combine ample fresh material from a few plants growing very close to each other; in order to collect viable samples from the freeze dried extracts. After collection the roots were cut into smaller pieces; approximately 1.0 cm^2. Approximately 121 g of these processed roots were added to 3.5 L of boiling tap water. At fixed time intervals (T_{00}, T_{05}, T_{10}, T_{15} and T_{20} minutes) 500 ml of the boiling liquid was removed from the pot. These volumes were separately filtered through Whatmann No. 1 filter paper, and the filtrate stored in appropriately labelled glass containers. The filtrates were frozen; where after they were dried via freeze drying (VIRTIS, United Scientific (PTY) Ltd.). The dried, concentrated extracts were used to prepare 50 mg/ml stock solutions to be used in the micro-dilution assays.

Time 0 (T_{00}) was considered the removal of a 500 ml volume within 15–20 seconds after adding the plant material to the boiling water. The other samples were collected at 5 minute intervals succeeding T_{00}. No additional water was added (no topping up) in order to concentrate the extract so that the highest possible yield of bioactive compounds could be collected.

13.2.3 Quantitative analysis by High Peformance Liqiud Chromatography

HPLC analyses were performed on a Shimadzu instrument (Shimatzu Corp., Kyota, Japan) equipped with a quaternary pump, an autosampler, column thermostat and a diode-array-detector (DAD) with a sampling frequency of 2 Hz. Chromatographic sample separation was carried out on a Discovery® Bio wide pore C_{18} column (4.0

mm i.d. x 25 cm) at 40 °C. Elution was performed with a flow rate of 1.0 ml/min. The binary mobile phase consisted of acetonitrile (A) ammonium acetate, 10 mmoL (B). The following gradient elution was employed: 10% A (2 min), 10–30% B (10 min), 30–45% B (50 min), 70–80% B (65 min) and 80–95% B (70 min). Subsequent to the running, the gradient was set back to 10% A and the system was allowed to reach equilibrium, where after an injection volume of 20 µl was used. Spectral data from all peaks were recorded over the 200–400 nm range, and integrated at 254 nm for alkaloids, 320 nm for phenolics, and 220 nm for terpenes.

13.3 RESULTS

The quantitative study of the terpene (λ = 220 nm), alkaloid (λ = 254 nm) and phenolic (λ = 320 nm) compounds present in *C. roseus* aqueous extracts was performed by HPLC-DAD. In this study the focus was on boiling-time dependent peak patterns that followed a similar trend to that observed in the previously reported minimum inhibitory concentration responses (MIC) against *N. gonorrhoea*.

Table 13.1 summarises the number of peaks observed at each of the mentioned wavelengths; including the pathogens tested together with their respective MIC values. The data set is categorised according to boiling time intervals ($T_{00–20}$). Table 13.1 further indicates that of the four pathogens exposed to aqueous extracts prepared from *C. roseus* roots, only *N. gonorrhoea* exhibited noteworthy inhibitory activity. Therefore, these were the only MIC responses compared to the crude extract HPLC-DAD analysis (Table 13.2); to explore and compare the phenolic, alkaloid and terpenoid levels with the previously reported antimicrobial responses.

CHAPTER 13

Phytochemical analysis of *Catharanthus roseus* crude aqueous extracts

Table 13.1: Effect of boiling on the phytochemical profile of crude extracts prepared from *Catharanthus roseus* roots.

Time interval	Pathogens	MIC (mg/ml)	Wavelength		
			*220 nm	**254 nm	#320 nm
			Number of peaks (n)	Number of peaks (n)	Number of peaks (n)
T_{00}	*N. gonorrhoea*	**0.78**##	165	113	43
	P. vulgaris	12.5			
	C. albicans	12.5			
	S. aureus	6.25			
T_{05}	*N. gonorrhoea*	**0.78**##	169	132	51
	P. vulgaris	6.25			
	C. albicans	6.25			
	S. aureus	6.25			
T_{15}	*N. gonorrhoea*	**0.78**##	182	155	58
	P. vulgaris	12.5			
	C. albicans	6.25			
	S. aureus	6.25			
T_{20}	*N. gonorrhoea*	**0.78**##	184	166	64
	P. vulgaris	12.5			
	C. albicans	6.25			
	S. aureus	6.25			

Wavelengths: terpenes*, alkaloids** and phenolics#; Significant antimicrobial responses ##.

Table13. 2: Chromatogram peaks identified in relation to the antimicrobial responses observed when *Neisseria gonorrhoea* was exposed to various aqueous *Catharanthus roseus* root extracts.

MIC (mg/ml)	**0.78****	**0.78****	**----**	**0.78****	**0.78****
HPLC peaks (phenolics, λ = 320 nm)					
RT*	**T_{00}**	**T_{05}**	**T_{10}**	**T_{15}**	**T_{20}**
23.801	1.436	1.342	1.137	1.189	1.190
25.790	5.622	6.444	5.747	5.722	5.616
37.716	1.742	1.633	1.479	1.434	1.516
42.229	0.781	0.705	0.678	0.725	0.732
45.865	21.758	25.070	24.538	25.916	26.441
HPLC peaks (alkaloids, λ = 254 nm)					
2.632	0.257	0.281	0.241	0.238	0.233
3.257	0.185	0.203	0.191	0.184	0.202
3.540	0.092	0.090	0.092	0.085	0.105
10.593	0.049	0.053	0.046	0.043	0.053
19.752	0.049	0.057	0.055	0.057	0.048
20.721	10.812	12.920	12.188	12.156	12.607
22.859	0.102	0.098	0.104	0.094	0.116
23.141	2.141	2.428	2.289	2.245	2.411
25.273	0.188	0.198	0.199	0.161	0.192
25.775	1.082	1.277	1.220	1.201	1.271
26.379	0.139	0.123	0.127	0.112	0.116
30.421	2.785	3.003	2.958	3.004	2.997
36.457	0.832	0.961	0.830	0.960	0.836
36.688	1.136	1.083	1.104	1.196	1.060
37.640	3.606	4.358	4.190	4.284	4.214
41.329	0.530	0.613	0.624	0.639	0.651
41.625	0.078	0.077	0.078	0.078	0.070

41.967	0.352	0.392	0.410	0.427	0.435
44.538	0.102	0.105	0.108	0.107	0.104
44.996	15.074	16.723	18.119	18.087	17.900
45.864	22.056	26.314	26.845	26.704	26.886
48.838	0.043	0.048	0.044	0.040	0.047
51.975	0.125	0.128	0.154	0.125	0.137
58.223	0.199	0.195	0.183	0.177	0.187
HPLC peaks (terpenes, λ = 220 nm)					
14.390	0.954	1.051	**----**	0.856	0.845
15.260	1.147	1.002	**----**	1.198	1.293

*RT = retention time obtained from respective chromatograms; Significant antimicrobial activity**. ----- indicates that no data set was available for that specific boiling period.

13.4 DISCUSSION

From a global perspective gonorrhoea remains one of the most prevalent sexually transmitted infections. If left untreated or inadequately treated serious health problems might arise. Amongst its most prominent complications are male epididymo-orchitis and pelvic inflammatory disease in females. The most notable adverse sequelae of PID include infertility and ectopic pregnancies. In South Africa, gonorrhoea causes 70–80% of the male urethritis cases (Lewis, 2009). This is a fairly common clinical condition that in symptomatic cases present with a combination of urethral discharge, burning on micturition and periodically dysuria (Ishihara et al., 2004; Sturm et al., 2004). In South Africa this pathogen accounts for almost 15% of the cases presenting with an abnormal vaginal discharge (Lewis, 2009). Traditional healers, in their inclusion of an abnormal discharge as a diagnostic criterion, therefore partially correspond with their modern medicine counterparts. Similarly, the disappearance of this discharge is more often than not considered as an indication of the efficacy of the herbal remedies prescribed by a traditional healer.

Herbal remedies are crude extracts primarily prepared in an aqueous medium. These extracts contain a magnitude of secondary metabolites such as terpenoids,

phenolics and alkaloid; compound groups known to contain principles with antimicrobial and anti-inflammatory potential. It is important to acknowledge the possible contribution of both antimicrobial and anti-inflammatory modalities in resolving the urethral discharge demonstrated in symptomatic gonorrhoea. From a physiological perspective the urethral discharge is as much a result of the exposure to an infective agent as it is an indication of the concomitant, underlying immune response towards the damage caused by these pathogens to the epithelial lining of the urogenital tract. Therefore, any secondary metabolite with microbicidal and/or anti-inflammatory potential is of tremendous therapeutic value in the fight against drug resistant pathogenic microbes infecting the human reproductive tract.

Catharanthus roseus was selected because it was previously reported that the Bapedi uses only the roots to treat a single ailment, *viz* gonorrhoea (Erasmus et al., 2012; Semenya et al., 2012). In effect this indicated that, according to these traditional healers the use of crude extracts from this species alleviated the symptoms; clearly proposing a possible antimicrobial/anti-inflammatory capacity. The fact that the Bapedi employ boiling times that varies from 5 to 20 minutes when they prepare these remedies, led to the emergence of the theory that extended boiling will alter the phytochemical profile as well as the antimicrobial efficacy. Our previous work (Chapter 10) found that all of the aqueous extracts prepared exhibited similar inhibitory activity on *N. gonorrhoea*, contradicting our initial theory that there should have been a fluctuation in the antimicrobial response. As a matter of fact these findings suggested that there is absolutely no need to boil this plant material at all! The current study is an extension of this, where the emphasis was on the changes that occurred in the phytochemical profile as boiling time increased. This was based on the perception that it was highly unlikely that the phytochemical profile remained unchanged throughout the boiling period, thus identifying secondary metabolite peaks that remained relatively constant throughout the boiling period might support their involvement in the antimicrobial response.

There is no MIC for T_{10} as the extract was lost during freezing when the container cracked. However, in the light of the consistent MIC levels detected, it was reasoned that phenolic, terpenoid and alkaloid peaks exhibiting a relative consistent

level (area%) throughout, might single out their involvement in eliciting this antimicrobial response. By applying this concept it was found that a number of secondary metabolites exhibited this trend. The most prominent group was the alkaloids (λ = 254 nm; n = 24), followed by phenolics (λ = 320 nm; n = 5) and lastly terpenoids (λ = 220 nm; n = 2). In essence this does not imply, per se, that the terpenoid and phenolic compounds are insignificant in the therapeutic value of this plant species. The fact that both of these groups are known to possess antimicrobial and anti-inflammatory potential (Polya, 2003) warrants further investigation into their identification and confirmation of a possible therapeutic application which might proof invaluable in the development of new antimicrobials.

In the present study the identification of so many possibilities from within the alkaloid ranks was anticipated. It is well known that *C. roseus* produces a magnitude (>150) of monoterpenoid indole alkaloids (Jaleel et al., 2009), a number of which have been found to be dimeric and bis-indole alkaloids (Gurib-Fakim, 2006). In support of our findings, Van Wyk et al. (2002) and Govindasamy and Srinivasan (2012) indicated indole alkaloids as the active principles involved in the antibacterial activity of *C. roseus*, even though *N. gonorrhoea* was not one of their evaluated pathogens. Future research interest, set against the increased emergence of antimicrobial resistance, regarding the specific alkaloid/s involved should focus on isolating them and evaluating their antimicrobial efficacy. The antigonococcal activity of crude extracts should also be compared to previously identified alkaloids; 19-S-vindolinine, vindolinine, tabersonine, ajmalicine, catharanthine and serpentine; all of them have been isolated from aqueous root extracts and might be involved in the antigonococcal activity of *C. roseus* (Ferreres et al., 2010).

It can be concluded that an increased boiling time did indeed alter the phytochemical profile of the aqueous root extracts; as phenolic, alkaloid and terpenoid presence all increased as boiling time progressed. Further analysis of the chromatograms identified alkaloids as the active antigonococcal principle. The fact that so many of the alkaloids were implied needs further investigation as to their identification, as well as the possible synergistic effects amongst them.

EPILOGUE

CHAPTER 14

14.1 INTRODUCTION

Exploring the healing powers of plants is an ancient concept, dating back some 5000 years, thereby supporting the perception that medicinal plants constitute the oldest and most widespread medicinal commodity (Nessler et al., 1985; Bechgaard, 1997). Many recent advances in the development of modern-day therapeutics originated from the *materia medica* used in traditional medicine (Balunas and Kinghorn, 2005).

Many people rely on traditional medicine for their health care requirements. Since traditional medicine is rooted in cultural and religious beliefs, it is not unusual to find that individuals who consult at modern health facilities also access folk medicine (Amusan et al., 2007). Traditional medicine has a long history of trial and error, which has resulted in the identification of particular plant species in the mitigation and/or complete curing of specific ailments. One of the most common components of a traditional healing system is called the “Doctrine of Signatures”, which dictates the selection of plants or plant parts based on their size, shape, colour or texture. Thus, according to this approach, the plants’ physical characteristics are indicative of its therapeutic use or potential (Kennett, 1976; Browner, 1985).

Along these lines it makes sense that modern day drug development would focus on the screening of natural products with a proven traditional record; an approach often referred to as “reverse pharmacology” (Wilcox et al., 2011). This would imply a more focused approach rather than random screening, which will ultimately save time and money in the quest for drug development. The appeal of such an approach is based on the perception that “substantial experience of human use increase the chances that a remedy will be effective and safe” (Patwardhan and Mashelkar, 2009).

14.2 TRADITIONAL MEDICINE IN SEXUAL HEALTH CARE

Sexual health incorporates much more than only the aspects related to STIs, even if these infections can negatively affect the overall sexual health status of an individual. In this study the sexual health care focus was on specific pathogens associated with STIs, as well as the antimicrobial efficacy of selected aqueous extracts prepared via different boiling protocols, used by the Bapedi in the treatment of STIs. However, all of this has limited application value if it is not placed in context by the addition of aspects such as the increased resistance of these pathogens to modern day antimicrobials, factors increasing the risk of attaining an STI, and ultimately an understanding of the role of the human reproductive tract in the acquisition of and resistance to microbial infections.

14.2.1 The human uro-genital tract: Immunological responses

The uro-genital tract of females and males are complex compartmentalized systems. The mucosa is distinct from other mucosal sites, such as those found in the gastro-intestinal tract and the respiratory system, as they do not possess inductive mucoepithelial sites (McDermott et al., 1980). In females a major site of infection and immune defense is the cervical transition sone. At this site the stratified squamous vaginal epithelium is transformed into the glandular columnar epiuthelium of the endocervix. Similarly, in males, the glandular columnar epithelium of the penile urethra is not nearly as impermeable as the stratified squamous epithelium of the penile skin and *fossa navicularis* (Pudney and Anderson, 2011).

These areas, that are more prone to infection, can activate a combination of the innate and acquired (adaptive) immune responses upon exposure to a foreign entity. Where the innate response is a more generalized reaction, acquired immunity is a result of the ability of the human body to develop extremely powerful specific immunity against particular invading agents. Unlike, the innate system that predominantly uses phagocytes, acquired immunity activates a special immune

system that employs antibodies (immunoglobulins) and activated lymphocytes to eliminate the invading pathogen (Guyton and Hall, 1996).

Innate immunity of the female reproductive tract involves the production of microbicidal mucus by the epithelial cells. These epithelial cells not only form a physical barrier against invading pathogens, they also express pattern recognition receptors (PRRs) which recognizes pathogen-associated molecular patterns (PAMPs) and as a result mediate the secretion of chemokines, cytokines and antimicrobial peptides. Adaptive immune responses consist predominantly of immunoglobulin G (IgG) and to a lesser extent IgA (Mestecky et al., 2010).

Immune response in the male reproductive tract is more complex as it is an immune priviledged site. A situation that occurs because of the presence of the blood-testis barrier, which results in the relative suppression of adaptive immunity and its concomitant, enhanced innate immune responses (Brotman et al., 2013). Similar to the female tract, the male urethral epithelial cells express PRRs and are implicated in antigen presentation. Regarding the acquired immune response, IgG is the key immunoglobulin found in seminal plasma. Furthermore, the abundant distribution of immune cells in the penile urethra suggests that it may be a major site of immune induction (Pudney and Anderson, 1995).

Understanding human immune responses is pivotal in phytomedicine. A magnitude of ethnobotanical and *in vitro* studies explored, and will continue to explore, the antimicrobial use and potential of plant extracts. Frequently it is concluded that these extracts exhibited no noteworthy (MIC < 1mg/ml), or very low antimicrobial activity, and could therefore not be validated as useful in the treatment of a specific ailment. Yet, specific plant / plant part / multi-plant extracts continue to be used in traditional medicine and reportedly remain effective in alleviating the symptoms. Disregarding the continued ethnobotanical use, further interest in that specific plant species and its use in the treatment of STIs is prematurely terminated. The fact that Rios (2010) in his comprehensive review on the effects of triterpenes on the immune system, illustrated that a plethora of research successfully addressed the immunomodulatory potential of triterpenes, should be a cause of concern when antimicrobial studies are prematurely discontinued. In the advent of increased microbial resistance to currently available antibiotics, phytopharmaceutical studies

into ethnobotanically identified and verified remedies should revisit analytical approaches employed.

14.2.2 Risk factors for attaining a sexually transmitted infection

The mere fact that sexually mature individuals partake in sexual activities puts them at risk of being exposed to causative pathogens involved in the etiology of STIs. However, a number of behavioral and demographic factors might contribute significantly to the risk profile of an individual. However, figures related to the worldwide incidence of currently curable STIs such as gonorrhea, chlamydia and syphilis cannot be considered as definitive. Confounding factors include the uncertainty regarding prevalence estimates, the duration of untreated infections and the average duration before commencement of treatment (Mabey, 2010). Add to this the fact that most STIs present asymptomatically, and that no reliable estimates pertaining the incidence of STIs in rural settings exist; then the future for STI management becomes a grim prospect.

The implication of demoghraphic factors and sexual behavior in the transmission and acquisition of STIs is not new. It was found that adolescents, aged 15–19 years, represents the age group at highest risk for attaining an STI, mostly because of their inclination to participate in risky sexual activities such as refusal to use condoms, multiple sexual partners and concurrent sexual relationships (Rosenberg et al., 1999; Hughes et al., 2000; Eaton et al., 2010). The gender disparity suggests that adolescent females are disproportionately affected when compared to males of a similar age.

Some socio-economic determinants support the fact that STIs are more common in poor populations, especially those residing in rural areas. What are some of the factors contributing to the increased risk reported amongst the poor? The vast distances travelled in the search of employment resulting in the migration of poor rural villagers into cities. It is equally true that a lack of education and poverty can drive females, and sometimes males, into the sphere of commercial sex networks. In addition to this, health education and the lack of appropriate health services often results in unneccesary delays in treatment (Mabey, 2010).

It is evident from the above that in South Africa a large percentage of our population residing in rural areas are at risk. Even though only a proportion of them present with symptoms, which will aid in the diagnosis by traditional healers, the infection rate amongst asymptomatic individual are of greater concern. These asymptomatic individuals appear healthy and will ultimately contribute to the ineffective treatment of STIs as they will keep on re-infecting their partners. This is disturbing as it is currently not known if and to what extent traditional healers insist on treating the partners of symptomatic individuals; as their modern medicine counterparts do insist on treating all parties involved. It is of the utmost importance to collaborate with traditional healers to establish the incidence of STIs among symptomatic individuals and to ascertain the identity of infective agents and co-infections.

14.2.3 Microbial resistance: A source of concern

The sexual health effects of increased antimicrobial resistance amongst pathogens associated with infections of the uro-genital tract is a constant source of concern. Rightfully so, as these pathogens are a major contributor to acute illness globally, and they continue to exhibit high prevalence rates in resource-limited countries, with reduced, but still substantial rates in some developed countries (WHO, 2011c).

The burden of infection, with approximately 444 million cases of curable STIs reported annually (WHO, 2011b), combined with the rapidly fluctuating antimicrobial susceptibility of some pathogens has serious implications for the treatment and control of STIs (Ndowa and Lusti-Narasimhan, 2012). It should be remembered that antimicrobial resistance has the potential to increase the burden of disease even further by extending the infection to more people, and also by progressively increasing the number of individuals with long-term sequelae. The development of resistance in virtually all clinically relevant pathogens highlights the urgent need for alternatives to conventional therapeutics.

Even though all pathogens tested, in the current work, are of clinical relevance *N. gonorrhea* exhibits the highest level of resistance to current therapeutics, and thus the biggest impact on sexual health aspects. Resistance to antimicrobials is a

common phenomenon amongst gonococci, and this group of pathogens has a rich history of effectively nullifying even the best antimicrobials available. Currently the mainstay of antigonococcal treatment is third-generation cephalosporins, specifically cefixime, which is orally administered in a single dose (Lewis and Marumo, 2009). However, numerous cases of emerging resistance to this class of antimicrobials have been reported globally (Forsyth et al., 2011; Unemo et al., 2011; Bolan et al., 2012). This creates quite a predicament, most natural product-based candidates that are currently under development are new-improved versions of old drugs (Guskey and Tsuji, 2010); these might be effective at first but they will ultimately suffer the same fate as their predecessors. Therefore, current drug requirements must focus on the development of *de novo* antibacterial drugs with completely new modes of action (Fernebro, 2011). At this point in time, plants with their overwhelming secondary metabolite profiles, can play a pivotal role in the identification, isolation and development new drug leads.

14.2.4 Antimicrobial efficacy of plants used in this study

This study, in accordance to other studies of a similar nature (Naidoo et al., 2013; Mulaudzi et al., 2011), considered MIC values of less than 1.00 mg/ml to be noteworthy and those <0.1 mg/ml to indicate excellent antibacterial activity. These guidelines are approached with caution as *in vitro* studies cannot predict the outcome of *in vivo* assessments, and it is possible that some of our aqueous extracts might exhibit improved antimicrobial activity when subjected to *in vivo* assessment.

Catharanthus roseus, a species exclusively used by the Bapedi to treat gonorrhea, showed potential in the inhibition of *N. gonorrhea*, with all MICs recorded at 0.78 mg/ml. This level of activity does not fall within the excellent or even clinically relevant range, yet its continued traditional use supports the notion that its therapeutic impact might involve far more than only antibacterial capabilities.

Ziziphus mucronata, combined with either *H. hemerocallidea* or *T. terrestris*, showed very poor activity against *N. gonorrhea*, *P. vulgaris* and *C. albicans*. The only noteworthy activity was detected when *Z. mucronata* was combined with *T. terrestris* (T_{10}). The fact that *Z. mucronata* featured in previous research

(unpublished data) as a common denominator in a number of multi-plant extracts, created the impression that this species might play an important role in contributing to the possible antimicrobial activity of these extracts. This theory could not be confirmed in our study, as individual and combined extracts exhibited poor antimicrobial activity.

Aloe marlothii leaf and root extracts exhibited quite a range of noteworthy activities against all pathogens tested. Results from this study indicated that this plant species is not only underutilized from a research perspective, its therapeutic value is completely underestimated. The fact that its T_{00} aqueous extract had a MIC <0.09 mg/ml should focus attention on further phytopharmaceutical analysis, to establish its value in future drug development.

14.2.5 Impact of boiling time variations

It is well known that traditional healers employ several techniques to acquire beneficial bio-active compounds from selected plant material. Cross-culturally the most common method is boiling, mostly in water; to yield a digestible plant-based decoction that is perceived as relatively safe (Halberstein and Davis, 1984; Johns and Kubo, 1988). This boiling period does not only assist in the mobilization of therapeutic substances, it also aids in the destruction and subsequent removal of toxic compounds. In theory this is very important as many plants contain poisonous substances that are potentially harmfull to human health; a phenomenon which is well supported by the fact that numerous deaths have been documented that resulted from accidental or unregulated consumption (Blackwell, 1990; Huxtable, 1990; Sundov et al., 2005).

Scientific support for the extraction procedure followed in our study can be found in the recent work by Vuong et al. (2013). They investigated the impact of temperature, time and water-to-leaf ratios on the yield of polyphenols from *Carica papaya* leaves. They concluded that the optimal extraction conditions for *C. papaya* aqueous leaf extracts were 20 minutes at 70°C with a water-to-leaf ratio of 100:7.5 ml/g. According to them, the yield of polyphenols decreased when the temperature was raised to 100°C, a phenomenon they attributed to thermally induced

decomposition. Other studies also reported a temperature dependent extraction relationship (Ballard et al., 2009; Alu'datt et al., 2011; Vuong et al., 2011).

In our study we could not determine a temperature dependent extraction relationship, as all extracts were boiled and the temperature was kept relatively constant throughout the boiling period. However, all aqueous extracts exhibited a time dependent extraction relationship for terpenoids, alkaloids and phenolics. It was interesting to note that *A. marlothii* leaf terpenoids peaked at T_{20}, for the roots it peaked at T_{05}, and when the plant parts were combined the terpenoid peak shifted to T_{10}. It is interesting as this trend could not be verified when *Z. mucronata* were combined with either *H. hemerocallidea* or *T. terrestris*. In general, almost all of the aqueous extracts reached peak alkaloid levels at T_{20}, irrespective of species or plant part used. In *A. marlothii* a trend, similar to that reported for their terpenoids, were observed for phenolics, and again no other species combinations exhibited this phenolic trend. Combining plant parts, or different species, seem to shift the maximum yield for phenolics to a boiling time in excess of 15 minutes.

Our results revealed that water can be an effective solvent for the extraction of terpenoids, alkaloids and phenolics from the plant species used in this study. Even if the extraction process seems to be time dependent. Add to this the fact that water is an inexpensive, safe, accessible and environmentaly friendly commodity when compared to other organic solvents. It is therefore not surprising that it is the solvent of choice in traditional medicine.

14.3 ROLE OF TRADITIONAL HEALERS

It is not entirely clear why traditional healers don't get the recognition they deserve for the role they play in primary health care. Common sense dictates that if investigations, into the medicinal value of natural products, play an important role in the identification of disease-relevant species; that traditional healers will be considered experts and consulted as such. However, in reality current partnerships with traditional healers tend to be a one-way street where they are used as sources of information only. It is interesting that the involvement of traditional healers pertain predominantly to the diseases treated, their diagnosis, the plants / plant parts/

combination of plants used, the preparation of extracts and the dosage and administration of these remedies. The key component in all of this is that in order to validate a traditional application; the specific plant, extract or dosage studied in the laboratory should be directly related to its traditional use (Vandebroek, 2013). In essence this implies that all aqueous preparations will not necessarily be equal; and therefore laboratory preparation of aqueous extracts in an effort to validate traditional usage should give attention to detail pertaining traditional preparation procedures.

With regard to the role that traditional healers can play in the provision of better sexual health care; various partnerships can be of great value. Nelson et al. (2010) indicated that in an effort to reach populations at increased risk, that creative partnerships must be developed to increase access to services and screenings. According to them a potential partnership could be forged between traditional healers and western trained clinicians. However, some evidence suggest that biomedical healthcare providers are only interested to collaborate on their terms, which entails unilateral referral where information flow is from healers to them (Madiba, 2010). Similarly, Kayombo et al. (2007) acknowledged the fact developing such collaboration is a tedious process and should be developed systematically. The following guidelines, adapted from Nelson et al. (2010) highlight aspects that should be considered when developing an initiative involving the collaboration between biomedicine health care practitioners and traditional health practitioners: recognize and embrace the fact that many South Africans utilize traditional health practitioners for prevention and acute care, collaboration between these groups in the development of culturally appropriate training and prevention strategies for traditional healers and their patients, continue to value and support the exchange of ideas between traditional and western medicine, and to explore the possible role of traditional healers in providing population-based health education.

14.4 RECOMMENDATIONS

This study promted the following recommendations:

- A more integrated involvement of traditional healers. Not only in conveying ethnobotanical and therapeutic information, but also as collaborators in

improving primary health care services in general, but more specifically that of sexual health services;

- The above supports the investigation of perceptions, beliefs and attitudes regarding intercultural healthcare. As a result this may provide better insight into the many forces that are involved in the unique interaction between traditional medicine and biomedicine, thus assisting in forging better integration of both (Vandebroek, 2013);
- More comprehensive ethnobotanical surveys that will include 'Retrospective Treatment Outcome Studies (RTO)', which will enable the retrieval of valuable information from traditional plant use. This approach, developed by Graz et al. (2005), adds clinical information and statistical analysis to the ethnobotanical method, thereby enhancing the possibility of identifying that single remedy that has the highest correlation with reported clinical recovery;
- The quest for the development of *de novo* antimicrobials demands the involvement of a diverse spectrum of specialist from various disciplines. However, since the improvement of human health is the centre piece of all research endeavours, the involvement of individuals with a comprehensive knowledge of human physiology is strongly recommended;
- From a primary health care perspective asymptomatic individuals infected with one or more STI increases the disease burden. It is unfortunate that inexpensive and accurate diagnostic tests, used for screening of populations at risk of infection, do not exist; therefore the appeal for the development of such diagnostic tests;
- Effective antimicrobial treatment is an essential part of addressing established infections, as a result antimicrobial resistance (AMR) surveillance should be encouraged. In South Africa monitoring AMR remains costly and logistically challenging (Maseko et al., 2012). As a result only a few dedicated in-country laboratories are involved in such surveillance, meaning that collected specimens are frequently transported over long distances. This in itself influences gonococcal viability and can skew the AMR data that is reported (Magooa et al., 2013). The fact that we are globally facing a situation where gonorrhea can become untreatable in the foreseeable future, demands urgent

attention from the South African Department of Health and Social Welfare to attend to the current South African regarding the effective control of this STI;

- The provision of feedback to local communities regarding issues related to the toxicology, side effects, dosage and standardization of herbal remedies in general, but also about specific plants / plant parts/ plant combinations used in the treatment of specific ailments.

14.5 FUTURE RESEARCH

From the onset the purpose of the current study was to evaluate the antimicrobial use of aqueous extracts, prepared from selected plant species; and to relate these activities to a specific compound group. Both of these aspects were successfully addressed. Regarding these findings, future research should focus on the following:
Results from this study support the possibility that the antimicrobial activities detected could be attributed to a number of compounds rather than a single bio-active entity. Therefore, research should focus on how the combination of these compounds enhances the antimicrobial efficacy and to identify the most relevant clinical combination. In addition aspects related to safety, toxicity and potential interaction with currently used drugs should be included. It should be considered that these extracts, those with noteworthy activity as well as those without, have the potential to alleviate STI symptoms via a non-antimicrobial mechanism. Therefore further evaluation of appropriate and relevant immunomodulatory and anti-inflammatory responses are required.

14.6 CONCLUSION

Plants, used by Bapedi traditional healers to treat conditions related to STIs, were avaluated in order to validate their application in traditional medicine. The first step was to confirm whether younger or more mature *A. marlothii* plants should be used in this study; findings supported the use of younger plants.

EPILOGUE

The preferred boiling intervals was employed as extraction parameter; and was subsequently found to significantly affect the extraction yield of alkaloids, phenolics and terpenoids, from the various plant species and plant parts involved in this study.

Combining chromatogram data with the antimicrobial findings illustrated the following. Evaluating the efficacy against Gram-negative pathogens, terpenoids present in *A. marlothii* extracts are most probably responsible for the detected antigonococcal effect; however, indications are that alkaloids might be responsible for the inhibition of growth detected for *P. vulgaris*. Antifungal activity of *A. marlothii* identified alkaloids present in the root and leaf extracts; in contrast to this, combining roots and leaves shifted the inhibitory activity for *C. albicans* towards the terpenoids. The antigonococcal and antifungal activities observed for *Z. mucronata*, and the combined, extracts reflect postively on the involvement of terpenoids. The antigonococcal activity exhibited by *Catharanthus roseus* correlates very well with the large number of monoterpene-indole alkaloids present in this species.

Many of these extracts showed potential as antimicrobials, thus validating them as traditional remedies. However, the absence of antimicrobial activity or very poor antimicrobial activity for some extracts does not necessarily nullify the use of these species in the treatment of STIs. Fernebro (2011) speculated on the possible contribution of these novel drugs, which are incapable of replacing antibiotics, as much needed complements to the drugs in use currently.

Accepting or rejecting the hypotheses of this study is based on the following:

- Plant parts from younger *A. marlothii* plants are better sources of phytochemicals than those from more mature plants;

  Phytochemical assessments comparing aerial parts collected from younger and older plants clearly indicated that the younger plants contained more bio-active compounds. It is therefore reasonable to argue that these younger plants are better sources of phytochemicals than more mature plants. This hypothesis is therefore accepted.
- Employing different boiling intervals will affect the bio-active profiles of aqueous extracts and establish the existence of time-dependent extraction relationships;

  The boiling time intervals employed in this study reflects those preferred by Bapedi traditional healers when they prepare decoctions. With the use of high

performance liquid chromatography a time-dependent extraction relationship was observed, as the number of peaks increased as boiling time increased. This hypothesis is therefore accepted.

- Multi-plant / multi-plant part aqueous extracts are more effective antimicrobials than single plant / single plant part aqueous extracts.

  The plant species and combinations employed in this study, and assessed for their antimicrobial potential; illustrated that this hypothesis has to be rejected. This decision is supported by the fact that *A. marlothii* roots exhibited better antimicrobial activity than when it was combined with leaves.

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