

Antimicrobial efficacy and toxicity profiles of conventional antimicrobial agents in combination with commercially relevant southern African medicinal plants

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Declaration

I, Zelna Hübsch, hereby declare that the work contained in this dissertation is my own original work, with all other sources of information acknowledged by means of a complete reference list. This dissertation is being submitted for the degree of Master of Pharmacy, at the University of the Witwatersrand, Johannesburg. This work has not been submitted for any other degree or examination at this or any other university.



Z. Hübsch

The 8 day of APRIL, 2014.

Dedication

*I dedicate this dissertation to my family and friends,
in particular my mother, Shari Ann Hübsch; sister, Verena Hübsch; partner, Wesley Lloyd
Booth and best friend, Muriel Huang.*

*Thank you for all the support, encouragement and unconditional love,
without which this work would not have been possible.*

Publications and presentations arising from this study

Conference presentations (oral)

Hübsch, Z., Van Vuuren, S.F., 2012. Antimicrobial efficacy of conventional antimicrobial agents in combination with commercially relevant, southern African medicinal plants. Academy of Pharmaceutical Sciences of South Africa conference, Rhodes University, Grahamstown, South Africa. September 12 – 15, 2012 (refer to Appendix A for abstract).

Hübsch, Z., Van Vuuren, S.F., Van Zyl, R.L., 2013. What are the implications of combining conventional antimicrobials with traditional medicinal plants? School of Therapeutic Sciences 2013 Research Day, University of the Witwatersrand (Faculty of Health Sciences), Johannesburg, South Africa. October 10, 2013 (**Awarded the Emerging Researcher Award for 2013**) (refer to Appendix B for abstract).

Hübsch, Z., Van Vuuren, S.F., Van Zyl, R.L., Cock, I.E., 2013. Can traditional medicinal plants have an effect on conventional antimicrobial therapies? Department of Botany and Plant Biotechnology Postgraduate Inter-university Symposium, University of Johannesburg, South Africa. October 29, 2013 (**Awarded Best Masters presentation**) (refer to Appendix C for abstract).

Papers for publication

Hübsch, Z., Van Vuuren, S.F., Van Zyl, R.L., 2013. Can rooibos (*Aspalathus linearis*) tea have an effect on conventional antimicrobial therapies? Submitted November 2013 to the *South African Journal of Botany* (refer to Appendix D for abstract).

Hübsch, Z., Van Vuuren, S.F., Van Zyl, R.L., Cock, I.E., 2013. Interactive antimicrobial and toxicity profiles of conventional antimicrobials with southern African medicinal plants. Submitted February 2014 to the *South African Journal of Botany* (refer to Appendix E for abstract).

Abstract

Traditional medicine plays a vital role in the cultural heritage of many South Africans, with at least 80% of the population relying on medicinal plants for their primary source of healthcare. It has been acknowledged that even in some of the finest hospitals in South Africa, people are often found to be using traditional medicine in combination with conventional treatment regimens. Despite the substantial use of medicinal plants in South Africa, limited information is available on the interactive properties between commercially relevant, southern African medicinal plants and conventional drugs. Furthermore, the potential for toxicity of these combinations has been sorely neglected. In orthodox medicine, antimicrobials such as antibiotics and antifungals are amongst the most commonly prescribed group of drugs. Therefore, there is a high probability for the concurrent use of these two forms of healthcare.

The aim of this study was to evaluate the interactive antimicrobial and toxicity profiles, when seven conventional antimicrobial agents (amphotericin B, ciprofloxacin, erythromycin, gentamicin, nystatin, penicillin G and tetracycline) were combined with the essential oils, aqueous and organic extracts of seven medicinal plants (*Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Aspalathus linearis*, *Lippia javanica*, *Pelargonium sidoides* and *Sutherlandia frutescens*).

The antimicrobial activity of the plant samples and conventional antimicrobials were evaluated, alone and in combination, using the minimum inhibitory concentration (MIC) assay against two yeasts, three Gram-positive and three Gram-negative bacteria. The combinations were further evaluated using the fractional inhibitory concentration (Σ FIC) assessment. Combinations demonstrating notable synergistic or antagonistic interactions were studied in various ratios (isobolograms).

Toxicity of the antimicrobials and plant samples were assessed, individually and in combination, using the brine-shrimp lethality assay (BSLA) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay on human kidney epithelial cells (Graham or HEK-293 cell line).

A total of 476 combinations were assessed for interactive antimicrobial potential. Of these combinations, 14.29% were synergistic, 7.56% antagonistic, 35.71% additive and 42.44% indifferent in nature. Some notable interactions were identified, such as the combination of *A. linearis* (aqueous and organic extract) with penicillin G, where a synergistic profile was most often seen against the three tested Gram-positive micro-organisms (*Staphylococcus aureus*, *Bacillus cereus* and *Enterococcus faecalis*), with Σ FIC values ranging from 0.01 (synergistic) to 0.94 (additive). Further notable interactions included *A. betulina* and *S. frutescens*, when combined with ciprofloxacin and tested against *E. coli*, which demonstrated a favourable synergistic profile, and could be of importance in the treatment of urinary tract infections.

In the BSLA, the notable interactions that were tested demonstrated no toxic effect. In the MTT cellular viability assay, the only combination demonstrating possible toxicity was that of *A. linearis* (aqueous and organic extract) in combination with nystatin (inhibitory effects of $73.76 \pm 3.36\%$ and $56.88 \pm 6.61\%$, respectively). Therefore, concurrent use should be cautioned and further *in vivo* studies warranted.

In conclusion, most combinations were found to be non-interactive, alleviating some of the concern related to the concurrent use of the two forms of healthcare. However, some notable combinations were identified, which could possibly have an impact on conventional treatment regimens. Therefore, further *in vivo* testing is warranted to support the *in vitro* findings.

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List of abbreviations

%	Percent
°C	Degrees Celsius
ΣFIC	Sum of the fractional inhibitory concentration
μg	Microgram/s
μl	Microlitre/s
μm	Micrometer/s
μ_{max}	Maximum specific growth rate
Abs	Absorbance
ADD	Additive interaction
AIDS	Acquired immunodeficiency syndrome
Amp	Amphotericin B
ANT	Antagonistic interaction
Aq	Aqueous plant extract
ATCC	American type culture collection
BC	Before Christ
BSLA	Brine-shrimp lethality assay
BP	British Pharmacopeia
BPC	British Pharmaceutical Codex
Caco-2	Human epithelial colorectal adenocarcinoma cell line
CFU	Colony forming units

Cip	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
CO₂	Carbon dioxide gas
Combo.	MIC of agents in combination
CV	Cardiovascular
CVS	Cardiovascular system
CYP450	Cytochrome P450
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGEG	Epigallocatechin-3-gallate
Ery	Erythromycin
EO	Essential oil
FBS	Foetal bovine serum
g	Gram/s
GC	Gas-chromatography
Gen	Gentamicin
GI	Gastrointestinal
GIT	Gastrointestinal tract
HAI	Hospital acquired infection or healthcare-associated infection
HEK-293	Human kidney epithelial cell line (Graham cells)

HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
hrs	Hours
IC₅₀	Concentration of test substance inhibiting 50% cell growth
IKS	Indigenous Knowledge Systems
IND	Indifferent interaction or non-interactive
Ind.	MIC of agents when tested individually
Int.	Interaction classification
INT	<i>p</i> -Iodonitrotetrazolium violet
IV	Intravenous
l	Litre/s
kg	Kilogram/s
LC₅₀	Concentration of test substance causing 50% brine-shrimp death
Merck Manual	The Merck Manual of Diagnosis and Therapy
MIC	Minimum inhibitory concentration
MIP	Minimum inhibitory percentage
mg	Milligram
ml	Millilitre
MRC	Medical Research Council
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MSDS	Material Safety Data Sheet
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

n	Number of experiments
NA	Not applicable, as no/insufficient essential oil or where MIC \geq
NCCLS	National Committee for Clinical Laboratory Standards
N_{max}	Reduced final cell concentration
NS	micro-organism not susceptible to antimicrobial
NT	control not tested
Nys	Nystatin
Org	Organic plant extract
PBS	Phosphate buffered saline
Pen	Penicillin G
Pty	Private Company
rpm	Revolutions per minute
SAMF	South African Medicines Formulary
S.D.	Standard deviation
S/M	Solvent/media control
STI's	Sexually transmitted infections
SYN	Synergistic interaction
Tet	Tetracycline
TSB	Tryptone Soya broth
TUT	Tshwane University of Technology
USP	United States Pharmacopeia
UTI's	Urinary tract infections
UV	Ultra-violet

V	Volt/s
v/v	Volume per volume
WHO	World Health Organisation
WITS	University of the Witwatersrand
w/w	Weight per weight

Chapter 1

Introduction

1.1. History of traditional medicine and plant use

Traditional medicine is practised throughout the world, with its existence far before that of modern scientific medicine, and comprises of the accumulated knowledge and skills acquired over generations, which allows for the maintenance of health, along with the treatment of illness (WHO, 2008). There are four types of existing herbal medicine, namely Asian, European, Neo-Western and Indigenous herbalism. European herbalism originated mostly from the Mediterranean populations. Asian herbalism originated primarily from India (Aryuvedic, Unani and Siddha), China (Wu-Hsing) and Japan (Kampo). Neo-Western herbalism is the combination of European and American herbalism and Indigenous herbalism can be described as that which is practiced by a specific ethnic or cultural group, where most often the knowledge and skills lie with a traditional healer (Elvin-Lewis, 2001).

Traditional medicine very often makes use of medicinal plants and herbs in the healing practice. The use of natural remedies in the treatment of various diseases originates from the ancient Greek, Egyptian and Chinese civilisations. These remedies were in use far before the existence of chemists synthesizing bioactive drugs (Ioannides, 2002). The Greeks and Romans were mainly responsible for the initial documentation of medicinal plant use. During the renaissance, the use of medicinal plants in holistic treatment regimens became a common practice, after which the science of “pharmacognosy” was identified (Kinghorn, 2001). Medicinal plants have always been highly valued by the various medical systems and are central in many traditional healing practices (Heinrich *et al.*, 2004; Van Wyk *et al.*, 2009; Silva and Júnior, 2010). Plants are regarded as being medicinal in nature if any part of the plant contains a substance or substances that can provide therapeutic effects (Sofowora, 1982).

1.2. Global perspective of traditional medicine and plant use

It has been estimated that at least 28% of plants existing on earth have been used medicinally (Akerlele *et al.*, 1991) and the World Health Organisation (WHO) has estimated that at least

80% of the population worldwide, makes use of medicinal plants as a primary source of medicine. Developing countries contribute mostly to this statistic (Evans, 1997; Cordell and Colvard, 2005). Plants have provided a source of some well-known medicines, such as quinine, atropine, opioids and taxol, to mention only a few. These drugs were very often the only treatments available for specific illnesses and are consumed worldwide, demonstrating the global importance of medicinal plants (Van Wyk *et al.*, 2009).

The use of medicinal plants and herbal remedies for the treatment of diseases has rapidly gained popularity throughout the world over the last few decades (Elvin-Lewis, 2001; Ioannides, 2002; Chinyama, 2009) and very often, is the only source of healthcare (Chinyama, 2009). This increased popularity can be attributed to various factors, including affordability and accessibility. The use of herbal remedies also allows a person to exercise control of their own healthcare (Elvin-Lewis, 2001) and hence provides people with a sense of autonomy and empowerment. Many people are also often disappointed by orthodox medicine, due to adverse effects experienced or a lack of effectiveness. This is often seen in the treatment of infections, where resistant microbes render conventional antimicrobials ineffective. Patients, who have lost faith in the conventional treatment options often turn to traditional remedies as an alternative (WHO, 2002). Plants have been considered safer and more affordable than synthetic drugs by many people (Iwu *et al.*, 1999). These perceptions have thus led to an increase in use of medicinal plants, in place of consulting conventional healthcare providers for conventional medication.

In Brazil, it was estimated that only 37% of the population makes use of commercial, conventional drugs. Therefore, more than half the population depend on natural products for their healthcare (Funari and Fero, 2005). In Germany, 80% of medical physicians have been found to prescribe herbal treatments (Gilani and Rahman, 2005). In Africa, traditionally used medicinal plants play a vital part in the cultural heritage, with an estimated 60% of the population consulting traditional healers (Chinyama, 2009; Van Wyk *et al.*, 2009).

1.3. South African perspective of traditional medicine and plant use

South Africa has a rich cultural heritage and the medicinal systems practised among the different cultures can vary considerably. Medicinal plants and herbs are widely used in traditional healing practices throughout South Africa, along with the rest of Africa. The use of medicinal plants as a means of treating disease is extremely pertinent among the indigenous populations in South Africa (Bhat and Moskovitz, 2009). These plants are often

the primary source of medicine in traditional healing practices and are therefore, a central part of many South Africans' daily lives. These plants are not only used for their medicinal properties, but for cosmetic and hygienic purposes too (Van Wyk *et al.*, 2009). It has been estimated that 12 to 15 million South Africans are reliant on traditional herbal medicines, which are prepared from over 700 indigenous plant species (Brandt *et al.*, 1995; Meyer *et al.*, 1996). In South Africa alone, there are over 200,000 traditional healers, with approximately 3,000 plants used in traditional healing practices. A massive informal and commercial market exists for medicinal plants in South Africa, with many people still mainly relying on these medicinal plants for their everyday healthcare needs (Van Wyk *et al.*, 2009).

Traditional healers kept no records pertaining to their knowledge of the medicinal plants used. The traditional healing remedies were mostly passed on from generation to generation, via word of mouth; resulting in very limited availability of information pertaining to traditional remedies (Van Wyk *et al.*, 2009). As such, collecting and recording of ethnomedical data on medicinal plants received major attention. The first published report on South African medicinal plants was by Pappe (1847). The publication included information on the medicinal plants that were most commonly used at the time. Another important publication on medicinal plants was that of Watt and Breyer-Brandwijk (1932), which provided a comprehensive report of medicinal plants used at the time. Hutchings *et al.* (1996) also contributed greatly toward the documentation of medicinal plants most commonly used by the Zulu nation. Van Wyk *et al.* (1997) and Van Wyk *et al.* (2009) further highlighted the field of medicinal plant research in South Africa.

It has been acknowledged on numerous occasions, that there is a need for further validation of these traditional medicinal plants, via scientific investigation, with priorities lying in the antimicrobial, antiviral, antihelmintic, antimalarial, anticancer potential and cardiac activity of medicinal plants (Sofowora, 1993).

1.4. Role of medicinal plants in westernised medicine

Plants have been used for the development of new modern medicines for decades (Hermann and Von Richter, 2012). Many well-known conventional drugs have been derived from plant sources, such as aspirin, quinine, morphine, codeine and atropine (Van Wyk *et al.*, 2009). According to Phillipson (2001), worldwide over 50% of the top 20 drugs most commonly used clinically, are derived from plants. To date, new conventional drugs are still being derived from plants. As quoted by Van Wyk *et al.* (2009), “medicinal plants are something of

the future, not the past”. There has been an increase in the number of phytomedicines which are entering the market throughout the world and this is not only limited to developing countries (Cordell and Colvard, 2005).

1.5. Commercialisation of medicinal plants

In South Africa, medicinal plants are most commonly sold in the informal traditional markets as crude, unprocessed plant material. Even though there is a considerable demand for these plants in the informal sector, very few have reached commercial success in the formal sector, such as in pharmacies. Even though plant use has not been fully accepted in orthodox medicine, plant and herbal remedy use has escalated and these remedies are becoming important commercial products, in their own capacity. Only sixteen South African medicinal plants have been partly or fully developed for commercial products. These include *Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Aspalathus linearis*, *Bulbine frutescens*, *Cyclopia genistoides*, *Harpagophytum procumbens*, *Hoodia gordonii*, *Hypoxis hemerocallidea*, *Lippia javanica*, *Mesembryanthemum tortuosum*, *Pelargonium sidoides*, *Siphonochilus aethiopicus*, *Sutherlandia frutescens*, *Warburgia salutaris* and *Xysmalobium undulatum*. Of these plants, *Aloe ferox* (Cape aloe), *Agathosma betulina* (buchu) and *Harpagophytum procumbens* (devil’s claw) have acquired the most international success (Van Wyk *et al.*, 2009; Van Wyk, 2011).

1.6. Infectious diseases

Bacteria, fungi and parasites are responsible for causing infectious diseases, which have become a major health concern. Infectious diseases are one of the main causes of morbidity and mortality among humans, particularly in areas of low economic status (Adwan *et al.*, 2009).

Even though numerous anti-infective agents have been discovered, infectious diseases still cause millions of deaths around the world. More than 25% of the 57 million deaths per year worldwide are as a result of infectious diseases. Lower respiratory tract infections, human immunodeficiency virus/acquired immunodeficiency virus (HIV/AIDS), diarrhoeal diseases, tuberculosis, malaria and measles are the infections most responsible for these deaths (Kolodziej, 2011). The threat is even higher in developing countries. This is due to the limited availability of medicines, the living environments that can be crowded and unsanitary, along with the increase in drug resistance (Okeke *et al.*, 2005). The increase in microbial

infections in recent years can also be attributed to the increase in the HIV/AIDS infection numbers, which renders those infected, more susceptible to other microbial infections (Chinyama, 2009).

1.7. Nosocomial infections

A nosocomial infection is a major complication associated with hospitalisation (Gaynes, 1997). It is also known as a hospital acquired infection or healthcare-associated infection (HAI). In 2002 it was found that 1.7 million HAI's occurred and was the sixth leading cause of death in America. The data similarly reflects that in Europe (Klevens *et al.*, 2007; Chopra *et al.*, 2008; Kung *et al.*, 2008). There are various contributing factors, such as the age of the patient, frequent use of antimicrobials and the type of surgical procedure undertaken (Swartz, 1994). The emergence of antimicrobial resistant microbes has limited the progress in the prevention and control of nosocomial infections (Emori and Gaynes, 1993). It is a major health concern, which needs to be addressed with the introduction of rational antimicrobial prescribing and use, along with the identification of new, more effective antimicrobial agents, to which no resistance has developed.

1.8. Conventional antimicrobial agents

A conventional antimicrobial agent is a substance, which can either be synthesised or naturally produced, and is capable of inhibiting the growth of (bacteriostatic) or killing (bactericidal) a microbe (Pelczar *et al.*, 1993; Brock *et al.*, 1994). Antimicrobial agents were discovered during the twentieth century and were central in curbing the increasing threat posed by infectious diseases on morbidity and mortality rates (WHO, 2002). Antimicrobial agents have, without a doubt, been one of the most important therapeutic agents in conventional medicine. Even though antimicrobials were an invaluable addition to modern healthcare, the success is increasingly being threatened by microbes developing resistance to them; which renders them ineffective (Clark, 1996).

1.9. The emergence of antimicrobial resistance

The increase in infectious diseases has led to increased antimicrobial use. The reliance on antimicrobial agents ultimately leads to increased antimicrobial resistance, which has resulted in more untreatable infections and higher morbidity and mortality rates (Cordell and Colvard, 2005; Van Vuuren, 2007). The overuse of antimicrobials is not solely responsible for the emergence of resistance, since the misuse of these agents has also aided in hindering

antimicrobial effectiveness. Due to this escalation in resistance, many patients are being left untreated. Disappointed with orthodox medicine, people often turn to traditional remedies (WHO, 2002).

There are three main categories for antimicrobial resistance, namely intrinsic (primary), acquired (secondary) and clinical resistance. Intrinsic or primary resistance occurs among the micro-organisms that have not even been exposed to the antibiotic or antifungal. Acquired or secondary resistance is seen when the microbes have been exposed to the antibiotic or antifungal during the therapeutic management of the infection, and is often due to a genetic mutation. Clinical resistance develops when there has been a failure in treatment, which could be affected by various factors, such as the immune status of the patients, the pharmacokinetics of the drug or the species of microbe being treated (O'Shaughnessy *et al.*, 2009).

The emergence of resistance toward conventional antimicrobials has become a major public health concern (Livermore, 2000), since previously treatable infections are now resulting in prolonged infections and higher mortality rates (Van Vuuren, 2007). This global concern has led to efforts being directed to finding solutions to the problem of antimicrobial resistant. One of the strategies to overcome this problem is the identification of new, more effective antimicrobial alternatives. Many studies are thus, being directed towards discovering new antimicrobials, to which no resistance has developed. Medicinal plants are one of the sources from which scientists are hoping to find new, more effective agents (Adwan *et al.*, 2009).

1.10. Natural products as antimicrobial agents

In order to find new, more effective alternatives for antimicrobial therapy, studies have been directed toward traditionally used medicinal plants (Van Vuuren, 2007). Medicinal plants, which over centuries, have proven to be successful in the treatment of infections among our ancestors, provide a promising source of new antimicrobial agents (Rabe and Van Staden, 1997; Cowan, 1999; Darwish *et al.*, 2002). The frequent traditional use of medicinal plants by a large proportion of people, for the treatment of infections, provides proof of the efficacy of plants as antimicrobial agents (Van Vuuren, 2007). It has even been acknowledged that antimicrobials derived from plants are rarely associated with side effects, and also have the ability to eliminate many types of infections (Chanda and Rakholiya, 2011).

Plant use in antimicrobial treatment was first reported in 3,300 BC in Europe, with the discovery of the “iceman”, a human body that had been preserved by the presence of a bracket fungus (Capasso, 1998). Plants, for centuries, have provided a source of treatment against microbial infections, with their use being well documented. Rios and Recio (2005) have even commented on the increased number of articles being published on the use of medicinal plants in antimicrobial therapies.

In order to validate the traditional use of medicinal plants in infection treatment, many studies have focused on determining the antimicrobial efficacy of plants throughout the world, and have included antimicrobial screening of plant extracts and essential oils (Rabe and Van Staden, 1997; Fabry *et al.*, 1998; Cowan, 1999; Dorman and Deans, 2000; McGaw *et al.*, 2000; Nascimento *et al.*, 2000; Huffman *et al.*, 2002; Duarte *et al.*, 2005; Eldeen *et al.*, 2005; Rios and Recio, 2005; Buwa and Van Staden, 2006; Van Vuuren, 2008; Chinyama, 2009). Some studies have even gone as far as testing isolated antimicrobial compounds from plants against various pathogens, with many of these studies being listed in a review by Cowan (1999) and later by Van Vuuren (2008). The antimicrobial properties of plants indigenous to South Africa have, however, been sorely neglected, in comparison to ethnobotanical research data available on plants from other continents and countries (Van Vuuren, 2007). Research focusing on discovering new antimicrobial alternatives of botanical origin and the scientific validation of effectiveness, could possibly lead to the acceptance of medicinal plants in western medicine. Even though research to identify natural products of botanical origin, that have the same or comparable antimicrobial efficacy as conventional antimicrobial agents, has become important; there is general consensus among the various studies that plant based antimicrobials possess a lower potency than conventional antimicrobials (Van Vuuren and Viljoen, 2011).

1.11. The concept of synergy

Another strategy to overcome antimicrobial resistance includes combination therapy. Various combinations have shown promising therapeutic outcomes in enhancing antimicrobial effectiveness of existing antimicrobials. This concept is known as synergy or potentiation. Synergy is a word derived from the Greek, *synergos*, which means to work together. It can be defined as a cumulative effect produced by an interaction between two different agents (Biavatti, 2009), where the cumulative effect is far greater than the effect of the individual agents (Berenbaum, 1978).

Pharmacologically, it has been known that combining drugs can often be beneficial. Therefore, multitherapy has gained popularity, as opposed to monotherapy, especially in the treatment of hypertension, cancer, malaria, HIV/AIDS and infectious diseases. The combination of conventional antimicrobials has been found to reduce the development of resistance of microbes toward the antimicrobials (Biavatti, 2009). This theory of combination therapy is not only common practice in conventional medicine, but in phytomedicine too. Plants have a very complex composition and the large diversity of secondary metabolites increases the likelihood for interactions. Plant combinations have been used for centuries, due to the beneficial effects of a combination (Biavatti, 2009).

As such, the concept could extend itself to the combining of conventional drugs with botanical products, for an enhanced effect. Synergy between combinations of conventional antimicrobials and plant extracts is a concept that has only recently been investigated in depth. Synergy could result in enhanced efficacy, reduced toxicity, decreased adverse effects, increased bioavailability, lower dose administration and reduced or delayed antimicrobial resistance (Cottarel and Wierzbowski, 2007; Inui *et al.*, 2007).

1.12. Combination studies of agents with antimicrobial properties

Many methods can be employed to formulate new alternatives with enhanced antimicrobial activity in order to combat antimicrobial resistance, such as using multiple antibiotics concurrently, or to combine already available antibiotics with phytochemicals in order to create a potentiating or synergistic effect (Sibanda and Okoh, 2007; Adwan *et al.*, 2010).

1.13. Combinations of natural products

Combinations of natural products, to provide an enhanced effect, have been common practice in traditional healing. Essential oils are commonly used in combination and have shown an enhanced effect (Van Vuuren and Viljoen, 2006; Suliman *et al.*, 2010). It has been acknowledged that essential oils, when used in combination, have been found to be much higher in inhibitory activity than standard antibiotics (Al-Bayati, 2008). Plant extracts have also been found to be used in combination. A study by Mabone (2013) identified various combinations of plant extracts which are used for the treatment of skin ailments, in traditional healing practices in South Africa. Often, these combinations were found to have an enhanced efficacy.

1.14. Combinations of natural products with conventional antimicrobial agents

Compounds in various plants have been found to be synergistic enhancers for conventional antimicrobials, even if the plant compounds do not possess antimicrobial activity themselves (Aiyegoro and Okoh, 2009). Many studies have evaluated the effects of combining natural products (essential oils or plant extracts) with conventional antimicrobials (cefuroxime, tetracycline, tobramycin, nystatin, amphotericin B and many more). These have been tested on a range of micro-organisms, including resistant microbes, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. In most cases, a synergistic interaction has been identified. Most of these studies have, however, focused on antibiotic combinations with common herbs, such as *Rosmarinus officinalis*, *Origanum vulgare*, *Thymus vulgaris*, *Mentha piperita* and *Melaleuca alternifolia* (Shin, 2003; Sato *et al.*, 2004; Braga *et al.*, 2005; Betoni *et al.*, 2006; Rosato *et al.*, 2007; Rosato *et al.*, 2008; Adwan *et al.*, 2009; Rosato *et al.*, 2009; Van Vuuren *et al.*, 2009; D'Arrigo *et al.*, 2010; Jarrar *et al.*, 2010; Silva and Júnior, 2010). The synergistic effect is represented by a reduced minimum inhibitory concentration (MIC) for the antimicrobial. The reduced MIC signifies an enhanced antimicrobial effect, which could ultimately render an ineffective antimicrobial, effective once again. This interaction has resulted in some plant extracts being defined as resistance modifying agents (Sibanda and Okoh, 2007). Both Adwan *et al.* (2010) and Van Vuuren and Viljoen (2011) proposed that the potentiating effect of plant extracts on conventional antimicrobials has been neglected and requires further investigation.

A study by Van Vuuren and Viljoen (2011) has summarized some combinations of plants with conventional antibiotics and the interactions which were noted. A review by Hemaiswarya *et al.* (2008) also provides a number of synergistic interactions that have been identified between natural products and antibiotics in the treatment of bacterial infections. A recent South African study on the combination of the ethanolic extract of *Ziziphus mucronata* with conventional antibiotics (tetracycline, chloramphenicol, amoxicillin and ciprofloxacin) found that more synergistic interactions (54.17%) occurred between the combinations than those of antagonism (1.39%) against clinically relevant bacteria (*Bacillus cereus*, *P. aeruginosa*, *Enterococcus faecalis* and *Escherichia coli*) (Olajuyigbe and Afolayan, 2013). Palaniappan and Holley (2010) also discovered the synergistic interactions between conventional antibiotics (ampicillin, tetracycline, penicillin, bacitracin, erythromycin and novobiocin) and natural antimicrobials (eugenol, thymol, carvacrol, cinnamaldehyde and allylisothiocyanate) when testing against resistant strains of *Salmonella typhimurium*, *S.*

aureus, *E. coli* and *Streptococcus pyogenes*, and it was acknowledged that some phytochemicals have the potential to reduce antimicrobial resistance. A few combination studies have investigated antimicrobials in combination with isolated phytochemicals, such as phenols, tannins and flavonoids (Sibanda and Okoh, 2007; Hemaiswarya *et al.*, 2008; Jayaraman *et al.*, 2010; Palaniappan and Holley, 2010), where again, many synergistic combinations were identified and attributed to the potentiating effect of natural products on conventional antimicrobials. No antagonistic interactions were identified in this study. Others have investigated the combination of conventional antimicrobial combinations with non-conventional antibiotics, such as tricyclic neuroleptics and antidepressants (Gunics *et al.*, 2000).

After conducting an intensive search of published literature, no studies were found that appeared to have investigated interactions between the southern African medicinal plants selected for this study and conventional antimicrobials when used in combination. These plants have been studied for their antimicrobial activity, as well as in polyherbal formulations (Suliman *et al.*, 2010); however, no evidence is available on their effects when in combination with conventional antimicrobials.

1.15. Prevalence of concurrent use of natural and conventional medicine

In southern Africa, traditional African medicine coexists with conventional medicine, as well as other alternative types of medicine, such as homeopathy, Ayurvedic and Traditional Chinese medicine (Van Wyk and Gericke, 2000). It has been acknowledged that the 60% of South Africans consulting traditional healers, very often use modern medical services concurrently (Van Wyk *et al.*, 2009). Even if western healthcare is available, traditional medicine still exists side by side with conventional medicine (Sindiga, 1994). Many people in southern Africa have been found to use both traditional and conventional medications concurrently (Van Wyk *et al.*, 2009), without knowledge of the potential interactions which may occur. It has also been acknowledged that even in some of the finest hospitals in South Africa, traditional medicine is found to be used by patients in conjunction with conventional therapies [personal communication, Dr M.G. Matsabisa, Director of the Indigenous Knowledge Systems (IKS) Health Unit, Medical Research Council (MRC)]. The practice of combining traditional or herbal remedies with conventional medicine has been found to be prevalent not only in southern Africa, but also elsewhere. In Israel, it was found that 49.40% of natural product consumers were also concurrently using conventional drugs (Givon *et al.*,

2004). A national survey performed in the United States of America, indicated that 72% of patients using herbal remedies were found to be using prescription drugs and 84% using over-the-counter medication in combination. It was also found that some patients preferentially combined these two forms of healthcare, with the belief that there would be a synergistic effect (Maizes and Dog, 2010). The major concern with concurrent use of these two forms of healthcare is the potential for natural product/herb-conventional drug interactions and the clinical consequences of these interactions (Fasinu *et al.*, 2012). This provides the basis to study southern African medicinal plants in combination with conventional medication, in order to identify any interactions which may potentiate or compromise a patients' therapy.

Many people believe that traditional medicines are safe for consumption due to the history of their use; however, that notion can no longer be valid. It has been found that many phytomedicines that are used in conjunction with over-the-counter or prescription drugs, result in many undesirable interactions and effects (Maizes and Dog, 2010).

1.16. Interactions between natural products and conventional drugs

The potential for the interaction between these two forms of therapy is worrisome and has become a major concern. Natural products are taken not only for the treatment of some diseases, but also for the prevention thereof. Hence, long-term consumption of natural products often occurs. This leads to an increased frequency of simultaneous consumption with prescription or over-the-counter conventional medicines and the likelihood for interactions. The concern escalates among the elderly, where natural products are a popular choice for the treatment of ailments and where conventional prescription medication is also in use. The general public is usually unaware of the possibility for interactions and their adverse effects, which further exacerbates the problem (Ioannides, 2002).

Natural products are very often poorly defined and may have variable ingredients to that stated on the packaging, which further contributes to possible interactions. Multiple drugs are very often used in combination, particularly in the elderly and chronically ill, where interactions very commonly occur. This situation could further be complicated by the concurrent use of natural products. Often, patients do not disclose the use of natural products to their healthcare providers, as they are not considered to be of any harm, but rather of nutritional value (Butterweck and Derendorf, 2012). A study by Klepser *et al.* (2000) revealed that no less than 40% of natural product users disclose their use of these medicines to their healthcare providers. The continuous practice of using commercial drugs along with

natural products has been attributed to the lack of knowledge of interactions, which becomes a major safety concern. The lack of reporting also contributes to the lack of information available on the interactions occurring (Eisenberg *et al.*, 1998; Barrett *et al.*, 1999; Butterweck and Derendorf, 2012; De Lima Toccafondo Vieira and Huang, 2012). The occurrence of these interactions has also been attributed to physicians and their limited knowledge pertaining to natural or herbal medicines and their potential for drug interactions and the impact thereof on the health of their patients. Another issue is the lack of proactively enquiring natural product or traditional medicinal use by the healthcare provider upon their consultation with patients (Clement, 2005; Ozcakir *et al.*, 2007; Fakeye and Onyemadu, 2008). The possibility of interactions between the two forms of healthcare has been identified as a serious healthcare concern in many hospitals throughout the world, with new regulations being implemented to ensure the full disclosure of traditional medicinal use during a consultation, before any conventional medicines are prescribed (Murphy, 1999). This, however, has not become common practice in South Africa yet.

Natural products have been found to interact with conventional drugs in a variety of ways. Sometimes the natural products interact at the site of absorption, thereby affecting the rate or extent of absorption of conventional drugs. Natural products can also interact with protein transporters and compete with conventional drugs for transporters or can interact with the liver enzymes responsible for metabolism of conventional drugs (Ioannides, 2002). Not only are herbal remedies active on their own, but they are also capable of potentiating or diminishing the therapeutic effects of conventional medication (Catania, 1998).

The interaction between conventional drugs and natural preparations or dietary supplements was first reported in the 1970s. There have been many reports on herb-drug interactions and it has been acknowledged that the ability of natural products to interfere with conventional drugs needs to be addressed in detail. The increased use of these products throughout the world, has led to an increased number of interactions being identified; where some have been fatal (Ioannides, 2002). A few of these interactions have been reviewed by Elvin-Lewis (2001), Ioannides (2002) and Fasinu *et al.* (2012). St. John's wort is a natural product that is commonly associated with herb-drug interactions, some of which have been mentioned in Table 1.1.

It has been acknowledged that future interactions can only be avoided if attention is drawn to research conducted in the area of pharmacology of drug interactions (Ernst, 2000).

Table 1.1. Common interactions between St. John's wort and conventional drugs (Ioannides, 2002).

Conventional drug	Interaction/effect of combination
Warfarin	Loss of anticoagulant activity, due to up-regulation of CYP450 liver enzymes.
Cyclosporin	Tissue rejection in organ transplants, due to up-regulation of CYP450 liver enzymes.
Indinivar/Nevirapine	Treatment failure in HIV/AIDS patients, due to up-regulation of CYP450 liver enzymes.
Oral contraceptives	Reduced efficacy leading to unwanted pregnancy, due to up-regulation of CYP450 liver enzymes.
Tricyclic antidepressants	Reduced efficacy leading to untreated depression, due to up-regulation of CYP450 liver enzymes.

CYP450 = cytochrome P450

The potential for interactions needs serious acknowledgment and requires scientific studies for confirmation on the presence or absence of such interactions. These findings also need to be relayed to the general public to ensure their safety.

Antimicrobial interactions are classified as synergistic, antagonistic, additive or non-interactive (indifferent) in nature. The implications of a synergistic interaction between traditional plants and conventional antimicrobials includes enhanced efficacy, thereby allowing lower dose administration, with reduced side effects and possibly reduced antimicrobial resistance (Van Vuuren and Viljoen, 2011). Phytochemical synergy could also assist in protecting the drugs from degradation, increased solubility or possibly increased membrane transport of conventional agents (Gurley, 2012).

In contrast, antagonistic interactions could, however, severely reduce the efficacy of conventional antimicrobials, thereby increasing the burden placed on healthcare systems. There have been many instances where natural products have been used concurrently with conventional medicine and severe reactions have been reported. Well characterised interactions have been summarised by Vickers *et al.* (2001), who emphasized that several interactions remain undefined and that if patients are taking conventional medication, that

natural products should be used with caution. Antagonistic interactions result in more of an agent being required to produce a particular effect when used in combination, than what is needed when used separately (Berenbaum, 1978).

Additive interactions provide an effect that is exactly equivalent to the effect of the individual agents when added together, that is no less nor greater in combination than when tested separately. Therefore, there is no advantage or disadvantage in combining them. Additive interactions usually occur between agents with similar mechanisms or sites of action. Interactions are classified as indifferent when no interaction occurs at all between the two agents within the combination and are thus known as non-interactive effects. The constituents of the combination have no effect on one another and therefore the effect of an indifferent interaction is one that is no greater than the effectiveness of the most active agent in the combination. Interactions are most often indifferent when the agents in the combination have different mechanisms or sites of action (Berenbaum, 1978, Odds, 2003). No advantage or disadvantage occurs due to the combination. Therefore, additive and indifferent interactions provide some relief for the concern related to concurrent use.

1.17. Toxicity of South African medicinal plants

Toxicology involves studying the adverse effects or toxic effects that chemicals have on living organisms (Katzung *et al.*, 2011). Toxic substances can have an effect on various organs (Botha and Penrith, 2008), such as the liver (hepatotoxicity) and kidneys (nephrotoxicity) (Larrey, 1994). Various other types of toxicity also exist, such as cardiac toxicity, reproductive toxicity and phototoxicity, along with carcinogenic, mutagenic and teratogenic effects. Toxicity is usually divided into topical and/or systemic effects (Burfield, 2000).

Surprisingly, a large number of plants that exist in the world contain toxic substances (Dowden, 1994). Toxins are usually a by-product or a mechanism developed whereby chemical substances provide a defensive system to deter insects and animals (Van Wyk *et al.*, 2002). Poisonous plants are commonly found in South Africa's indigenous flora and were found to be responsible for 6.5% of poisoning cases at the Poison Unit of the Charlotte Maxeke Johannesburg Academic Hospital, formerly known as Johannesburg General Hospital (Van Wyk *et al.*, 2002). Poisonings due to plants are mostly intentional, in the case of a suicide attempt, or accidental, mostly in the case of curious children. Medicinal plant use in traditional healing practises rarely cause poisonings (Van Wyk *et al.*, 2002). However, all

plants are known to be toxic if consumed in large enough quantities or if the plants have been misidentified (Van Wyk *et al.*, 2002; Liu, 2005). The chemical constituents vary greatly between the different parts of the plant, such as the leaves, roots, seeds, bark or fruit. This variation in chemical constituency can result in one part of the plant being safe for human consumption, whereas another part highly toxic, which can also contribute towards unintentional poisoning. Often, methods are employed during the preparation of the traditional remedy for the neutralizing of toxins, such as burning until a certain colour is apparent, or boiling the preparation (Van Wyk *et al.*, 2009). Plants that are poisonous at high doses can still be used therapeutically at lower doses, but must be administered with extreme caution to prevent any toxic effects (Botha and Penrith, 2008).

There is a general misconception amongst people that natural products are safe and far less toxic than pharmaceutical formulations. In the Western populations there is a definite notion that 'natural' is better than 'chemical' or 'synthetic'. The belief that traditional remedies are devoid of risk further prompts the consumer to use these remedies rather than conventional drugs (Ioannides, 2002). Natural products still have the potential for severe interactions and are not at all devoid of toxicity (Hermann and Von Richter, 2012; Markowitz and Zhu, 2012). Traditionally used, medicinal plants on their own are potentially toxic, as demonstrated in a review by Fennel *et al.* (2004). It has also been acknowledged that the toxicity of plants could be exacerbated when used in combination with conventional medicines (Fasinu *et al.*, 2012).

Since it is known that many plants produce toxic substances as a defence mechanism, it is necessary for toxicological evaluation of medicinal plants during the scientific investigative process, in order to ensure the safe use of medicinal plants (Moolla, 2005; Cavalcanti *et al.*, 2006; Bussmann *et al.*, 2011). Most herbalists have written about toxic plants (Thompson, 1931). However, scientific documentation of adverse effects and toxic levels of plants is lacking (Elgorashi *et al.*, 2003; Fasinu *et al.*, 2012).

With most of the studies to date focusing only on the testing of the antimicrobial activity of combinations, the identification of possible toxicity of these combinations has been neglected. Many studies have, however, acknowledged the need for extensive toxicological studies, not only of the combinations, but of the individual plants too (Fennel *et al.*, 2004; Adwan *et al.*, 2009).

1.18. Toxicity of conventional antimicrobials

All conventional drugs are also considered to possess some adverse or toxic effects. These unwanted effects often result in the general public being dissatisfied by conventional drugs and hence turning to traditional remedies (Ioannides, 2002). Antimicrobial agents within the same class share common adverse or toxic effects. Since these agents are mostly metabolized and removed from the body via the liver and kidneys, often hepatotoxicity and nephrotoxicity occur. Some other common adverse or toxic effects caused by antimicrobial therapy include gastrointestinal (GI) effects, neurotoxicity, cardiotoxicity, bone marrow toxicity, photosensitivity, ototoxicity and hypersensitivity effects. The extent of toxicity is most often dependant on the size of the dose, route of administration and the duration of therapy. Larger doses and longer durations of use have the potential risk to result in increased toxic effects (Merck Manual, 2006; SAMF, 2012).

1.19. Overview of the study

1.19.1. Aim and objectives of the study

Very limited scientific information is available on the interactions that may occur when conventional antimicrobial agents are combined with some of southern Africa's most commonly consumed medicinal plants. Interactive profiles could have a considerable effect on conventional treatment regimens, particularly since most patients do not report traditional medicinal use to healthcare providers. Therefore, the purpose of this study was to evaluate the interactive antimicrobial profiles (synergistic, additive, non-interactive and antagonistic interactions), when six commercially relevant, southern African medicinal plant samples (*Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Aspalathus linearis*, *Lippia javanica*, *Pelargonium sidoides* and *Sutherlandia frutescens*) (Appendix F.1 – F.7) were combined with seven conventional antimicrobial agents (amphotericin B, ciprofloxacin, erythromycin, gentamicin, nystatin, penicillin G and tetracycline) (Appendix G.1 – G.7) and tested against various pathogens.

The seven medicinal plants selected for analysis in this study are included in the list of the sixteen commercialised South African medicinal plants. These plants are also included in the 350 medicinal plants classified as the most commonly used and traded medicinal plants in the informal markets of South Africa (Van Wyk *et al.*, 2009).

Most studies on South African medicinal plants mainly focus on the antimicrobial activities of individual plants and very few studies have been undertaken (in respective comparison) on plants in combination. Furthermore, the toxicity of these plants and combinations with conventional drugs has been sorely neglected. Therefore, selected plants for this study were also assessed for toxicity, individually and in combination. A schematic representation for the outline of the project has been provided (Figure 1.1).

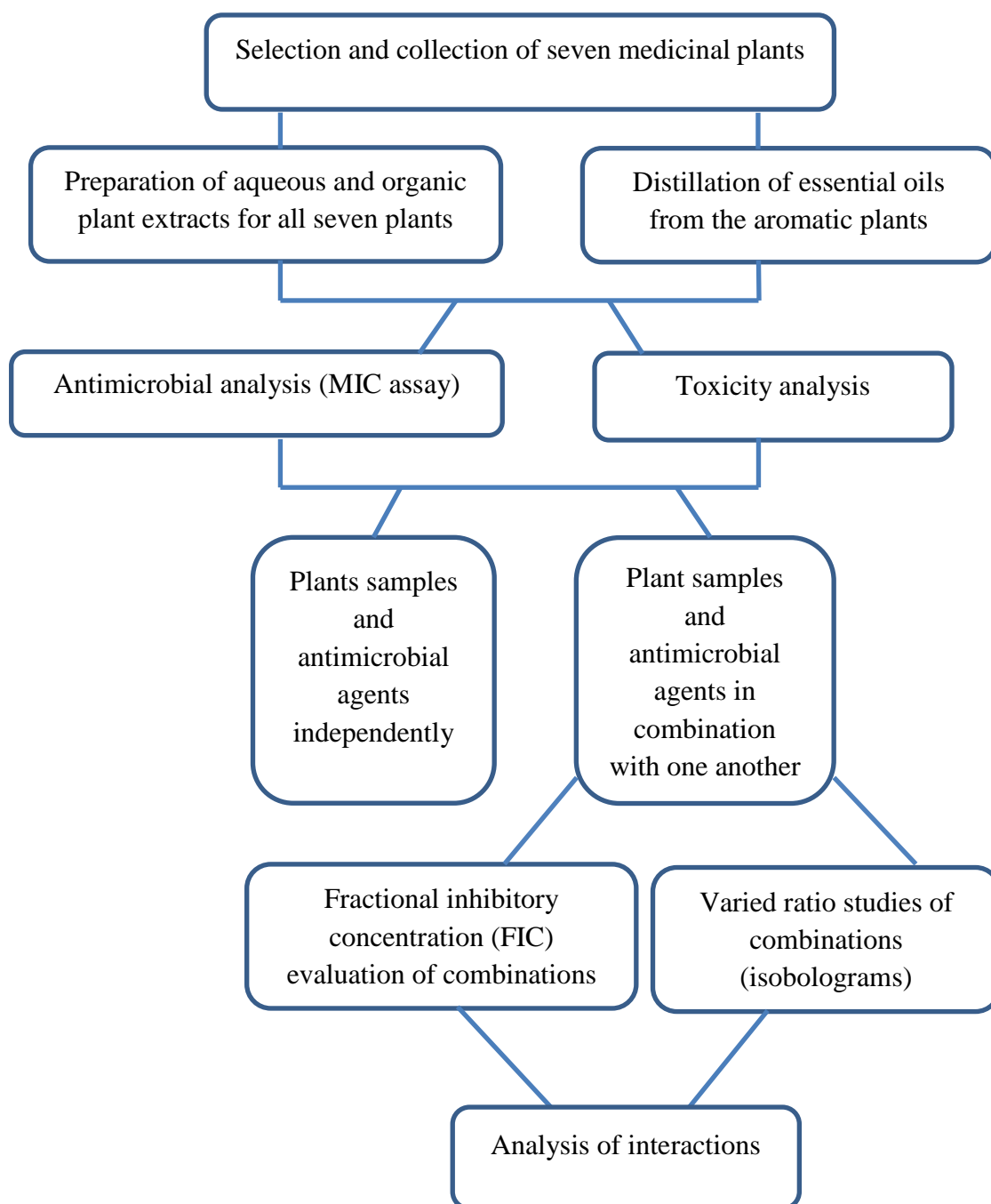


Figure 1.1. A schematic representation of the outline of the investigation.

To achieve these aims, the following objectives have been fulfilled:

1) Preparation of the plant samples:

- Sourced relevant plant material to be studied.
- Distilled the essential oils from selected aromatic plants.
- Prepared the aqueous and dichloromethane: methanol (1:1) plant extracts.

2) Determined the antimicrobial activity of samples using the MIC assay:

- Determined the antimicrobial activity of the selected plant samples (essential oils, aqueous extracts and organic solvent extracts) and conventional antimicrobial agents individually, against eight pathogens.
- Determined the antimicrobial activity of the selected plant samples in combination with conventional antimicrobial agents, against eight pathogens.
- Assessed the interactions by determining the sum of the fractional inhibitory concentration (Σ FIC).
- Where synergy or antagonism was observed in the Σ FIC assessment, various ratios of plant and conventional antimicrobial in combination were further evaluated for interactive antimicrobial effects (isobologram analysis), against the specific pathogen.

3) Determined the toxicity of the plant samples, as well as their combinations with conventional antimicrobials, using both the brine-shrimp lethality assay (BSLA) and the human kidney epithelial (Graham or HEK-293) cells in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay.

Chapter 2

Materials and methodology

2.1. Plant selection

In order to identify the most commonly used southern African medicinal plants, a literature review was conducted (Watt and Breyer-Brandwijk, 1962; Van Wyk, 2008; Van Wyk *et al.*, 2009; Van Wyk, 2011). Two reviews by Van Wyk (2008; 2011) outlined the most commercially relevant southern African medicinal plants and their potential use in the development of new medicinal products. As such, these reviews were used as a primary guide for this study for the plant selection process. All plants selected are included in the 350 species classified as the most commonly used and traded medicinal plants (Van Wyk *et al.*, 2009) and are therefore considered among some of the most important southern African medicinal plants for commercialisation (Van Wyk, 2008). The plants selected for analysis in the study included *Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Aspalathus linearis*, *Lippia javanica*, *Pelargonium sidoides* and *Sutherlandia frutescens*. These plants have already been partly or fully commercialised (Van Wyk, 2011), which could ultimately result in the practice of combining these remedies with conventional medicine, due to the accessibility and availability.

2.2. Plant material collection

Aloe ferox (voucher SVV-173) and *A. afra* (voucher SVV-172) were collected from the Walter Sisulu National Botanical Gardens, Gauteng. These plants were identified and harvested under the guidance of Andrew Hankey, Associate Curator, South African National Biodiversity Institute. *Agathosma betulina* (batch VV 01/13/02/12) was purchased from the commercial trader, S Chicken Naturals, Cape Town. *Aspalathus linearis* (4 kg super grade, pasteurised, fermented leaves) was donated by Rooibos (Pty) Ltd, from the Clanwilliam region of Cape Town. The leaves were provided in the form in which the tea is commercially sold, ensuring that the plant samples were as closely related to that which is used by consumers. *Lippia javanica* (voucher SVV-174) was identified and collected by Associate Professor S.F. Van Vuuren from the wild population in Fairlands, Johannesburg.

Pelargonium sidoides (batch 0212105) and *S. frutescens* (batch 0312010) were purchased from Parceval (Pty) Ltd Pharmaceuticals, Cape Town. Certificates of analysis were received from Parceval (Pty) Ltd Pharmaceuticals for these two plants, providing proof of their purity. All plant harvesting occurred during the warm summer months and the plant material was received at the University of the Witwatersrand (WITS) in March 2012. The plant parts to be analysed for the study were selected to be most closely related to the parts traditionally used.

2.3. Preparation of plant samples

Traditional medicinal plants are prepared and consumed in a variety of ways. For example, infusions (herbal teas), decoctions and alcoholic tinctures usually for oral consumption; boiling of aromatic plant material for the inhalation of volatile substances; and then poultices, or even infusions and decoctions, for topical application (Van Wyk *et al.*, 2009).

Infusions are prepared by submerging macerated plant material in cold or boiling water. The water is then administered orally, which is believed to contain the active ingredients of the plant (Van Wyk *et al.*, 2009). Decoctions are similar to infusions, however, are prepared from harder plant material, like the roots of a plant, and so require longer durations of boiling for the extraction of the active substances to occur. The plant material is most commonly boiled in water, but can also be boiled in any other solvent (Von Koenen, 1996). Tinctures are also a liquid preparation which would be administered orally. Tinctures are prepared by submerging macerated plant material in an alcoholic liquid, which differentiates it from an infusion or decoction, where water is most often the solvent. Some medicinal plants, particularly if used for respiratory complaints, are administered via inhalation methods. Plant material is boiled and the steam inhaled, allowing for the volatile ingredients to be inhaled, coming directly into contact with the respiratory tract lining. Sometimes the plants can also be burnt and the smoke inhaled (Van Wyk *et al.*, 2009). A poultice is a heated, macerated mass of plant material, softened by water, and applied directly on the affected area of the skin as either a hot or cold compress (Hutchings, 1996; Van Wyk *et al.*, 2009).

In order to mimic the antimicrobial effects that an aromatic plant could exhibit when the volatile substances are administered via inhalation, this study undertook to distil the essential oils from the aromatic plants. Similarly the effects provided by a tincture of the plant were mimicked in this study by preparing organic extracts. Aqueous extracts were also tested to

mimic the antimicrobial effects of a plant when consumed as an infusion or decoction or when applied topically as a poultice.

2.3.1. Preparation of essential oils (hydro-distillation)

Essential oils were distilled from the aromatic plants, which included three of the seven selected plants, namely *A. betulina*, *A. afra* and *L. javanica*. The aerial parts of *P. sidoides* also possess essential oils, however, the roots (tubers) were only analysed in this study, from which insufficient essential oil could be distilled. The tubers of the root of *P. sidoides* are the most commonly used part of the plant in medicinal preparations, therefore, only this part of the plant was analysed in the study.

The method of steam distillation (hydro-distillation) was employed, using a Clevenger-type apparatus (Figure 2.1). Round bottom flasks, with a five litre capacity, were packed tightly with fresh, aerial plant material (approximately 1 – 2.5 kg per flask) and approximately 800 ml of distilled water was added to the flask. The plant material and water was then heated using a heating mantel, which allowed for the release of essential oils in the vapour form. The steam and essential oil vapour was then cooled and returned to liquid state, using a condenser with cool water running through it. The distillation process continued for three hours at 100 °C. The condensed essential oils naturally separated from the condensed water due to differences in density with the essential oil layer above the water. This allowed for easy collection of the essential oil once the water had been siphoned off. The essential oils were collected in amber, glass gas-chromatography (GC) specific vials with tight sealing lids (Macherey-Nagel) to prevent evaporation, and stored at 4 °C until further analysis (Van Vuuren, 2007).



Figure 2.1. Essential oil distillation for *A. afra*, using a Clevenger-type apparatus.

2.3.2. Organic extract preparation

Organic extracts were prepared for all seven selected plants. Plant material was left to completely dry at room temperature for approximately seven days, after which it was ground into a fine powder using the high speed Fritsch Pulverisette grinder (Labotec). The ground plant material (approximately 20 g per 250 ml bottle) was submerged in a solvent system consisting of methanol and dichloromethane, which were combined in equal ratios (1:1). Methanol ensured the extraction of non-polar compounds, whereas dichloromethane ensured the extraction of polar compounds within the plant material. The ground plant material, submerged in the solvent system was left for 24 hours at 37 °C, in a platform shaker/incubator (Labcon), to allow for the extraction of compounds. Thereafter, the liquid was filtered and the filtrate left in open glass bottles, in a fume hood for the complete evaporation of solvent, leaving behind the solid extract. After evaporation, the organic extracts were stored in sealed, sterile glass bottles at room temperature until further analysis.

2.3.3. Aqueous extract preparation

Aqueous extracts were prepared for all seven selected plants. The aqueous extracts were prepared by submerging the ground plant material in warm, sterile distilled water, which was left in a platform shaker/incubator for 24 hours at 25 °C. The liquid was then filtered and the filtrate stored at -80 °C before lyophilisation (Virtis). Lyophilised aqueous extracts were left under ultra-violet light overnight to ensure the elimination of any microbial contamination. The aqueous extracts were then stored in sealed, sterile plastic bottles at room temperature and protected from light until further analysis.

2.4. Percentage yield of plant samples

The percentage yield was calculated for all the aqueous and organic extracts, as well as for the essential oils. The percentage yield was calculated by dividing the total weight of extract or essential oil obtained by the total weight of plant material used in the preparation of the extract or essential oil, which is known as the biomass. This value was then multiplied by one hundred to obtain a percentage weight per weight (w/w) (Table 2.1).

Table 2.1. Percentage yield values for all the plant samples investigated.

Plant	Plant part used in analysis	Percentage yield (% w/w)		
		Essential oil	Aqueous extract	Organic extract
<i>Agathosma betulina</i>	Leaves	1.54	1.43	4.80
<i>Aloe ferox</i>	Leaves	NA	4.14	2.99
<i>Artemisia afra</i>	Leaves and twigs	0.32	9.91	8.16
<i>Aspalathus linearis</i>	Leaves	NA	1.37	1.98
<i>Lippia javanica</i>	Leaves	0.69	8.31	11.16
<i>Pelargonium sidoides</i>	Roots (tubers)*	NA	7.84	3.18
<i>Sutherlandia frutescens</i>	Leaves	NA	11.42	5.89

NA = plant not aromatic in nature and hence no essential oil could be distilled; * = Even though *P. sidoides* is classified as an aromatic plant, the aerial parts possess majority of the essential oil, and thus insufficient essential oil could be distilled from the roots of the plant for analysis in this study.

2.5. Antimicrobial analysis

The minimum inhibitory concentration (MIC) assay was used to evaluate the antimicrobial activity of the plant samples and the conventional antimicrobials independently, followed by evaluation in combination. The guidelines for the micro-titre plate method, to determine the antibacterial activity of plant samples were in accordance with methods by Eloff (1998). The Clinical and Laboratory Standards Institute (CLSI) guidelines (2012) were followed when analysing antimicrobial (antibiotic and antifungal) samples.

2.5.1. Microbes used for analysis

To represent the three main classes of micro-organisms, three Gram-positive bacteria; *Staphylococcus aureus* (American Type Culture Collection (ATCC) 25923), *Enterococcus faecalis* (ATCC 29212) and *Bacillus cereus* (ATCC 11778), three Gram-negative bacteria; *Klebsiella pneumoniae* (ATCC 13883), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27858), and two yeasts; *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 14116) were included for analysis. The pathogens were selected on the basis of their prevalence in nosocomial infections or otherwise known as HAI. The ten most common pathogens that are responsible for 84% of all HAI's include coagulase-

negative staphylococci (15%), *S. aureus* (15%), *Enterococcus* species (12%), *Candida* species (11%), *E. coli* (10%), *P. aeruginosa* (8%), *K. pneumoniae* (6%), *Enterobacter* species (5%), *Acinetobacter baumannii* (3%) and *K. oxytoca* (2%) (Hidron *et al.*, 2008). Six of the eight pathogens selected for testing in this study appear in this list. The sites of most nosocomial infections include the urinary tract, lower respiratory tract, surgical wounds, the bloodstream and other areas, such as the gastrointestinal tract (GIT) and skin (Weinstein, 1998). The medicinal plants selected for analysis are very often used traditionally for the treatment of infections at these particular sites (Appendix F.1 to F.7). All micro-organisms were cultured in Tryptone Soya broth (TSB) (Oxoid), which was prepared to the required concentration (30 g/l), in accordance with the instructions from the manufacturer (dissolving weighed media powder in distilled water). The media, after autoclaving (Butterworth) at 121 °C for 15 minutes was left at room temperature to ensure sterility. Once sterility had been confirmed by the absence of turbidity, the media was stored at 4 °C, until needed. The micro-organisms were kept viable by sub-culturing on a weekly basis. Streak plates were prepared to ensure the purity of the culture, as well as for isolation of pure colonies for sub-culturing. The bacteria were incubated at 37 °C for 24 hours and the yeasts at 25 °C for 48 hours. A waiver for the use of these micro-organisms was granted by the WITS Human Research Ethics Committee (Reference W-CJ-130726-1) (Appendix H).

2.5.2. Antimicrobial and plant sample preparation

The selected conventional antimicrobials included erythromycin (potency of ≥ 850 µg/mg), gentamicin (potency of 600 µg/mg), nystatin (potency of $\geq 4,400$ United States Pharmacopeia (USP) units/mg), penicillin G (potency of 1440 – 1680 units/mg), tetracycline [$\geq 95\%$ High-performance liquid chromatography (HPLC)], ciprofloxacin ($\geq 98\%$ HPLC) and amphotericin B (80% HPLC), which were all purchased from Sigma-Aldrich (South Africa). The antibiotics and antifungals were prepared in sterile distilled water, to a concentration of 0.01 mg/ml and 0.1 mg/ml, respectively. The antifungal, amphotericin B, was dissolved in 1% (v/v) dimethyl sulphoxide (DMSO) in sterile water, to aid dissolution.

The plant samples (essential oils, aqueous and organic extracts) were all prepared to a concentration of 32 mg/ml. The essential oils and organic extracts were re-suspended in acetone, whereas the aqueous extracts were re-suspended in warm sterile distilled water. Water, heated to 30 – 40 °C, was used to aid the dissolution process of the aqueous extracts.

2.5.3. Minimum inhibitory concentration plate preparation

Each well of the micro-titre plate was filled with 100 µl of sterilized distilled water. The individual plant samples (32 mg/ml) and conventional antimicrobials (0.01 mg/ml for antibiotics and 0.1 mg/ml for antifungals) were then introduced into the wells of the first row (A), as 100 µl for individual samples. All samples and their combinations were tested in duplicate for accuracy or in triplicate where variance was observed.

The serial doubling dilution method was employed after all the necessary samples had been added to the first row of wells. The prepared micro-titre plates were then inoculated with the relevant pathogens, with each inoculum having a size of approximately 1×10^6 colony forming units (CFU)/ml (Van Vuuren *et al.*, 2008). Plates, sealed with a sterile adhesive sealer, were then incubated at 25 °C for 48 hours and 37 °C for 24 hours for yeasts and bacteria, respectively. Figure 2.2 provides a graphical representation of a completed MIC plate and the steps involved in the plate preparation, together with the interpretation of results.

After incubation, 40 µl of the colour indicator, 0.40 mg/ml p-iodonitrotetrazolium violet (INT; Sigma-Aldrich), was added to each well, which turned purple-pink in the presence of microbial growth (Figure 2.2). The MIC was therefore defined as the lowest concentration of a test substance with antimicrobial activity needed to inhibit the growth of a test micro-organism. Therefore, the first well within a column of dilutions (reading from the bottom of the plate upward), absent of the pinkish-purple colour (Figure 2.2), was taken as the MIC (Eloff, 1998). It is important to note that the colour change was not always easily apparent and results needed to be read with an additional light source to identify wells with a very pale pink colouring, indicating limited microbial growth (Figure 2.2). The antimicrobial activity of a sample could then be classified according to the MIC value. A summary of classifications seen in previous antimicrobial studies was compiled (Table 2.2), in order to determine an appropriate scheme for classification in the current study (Table 2.3).

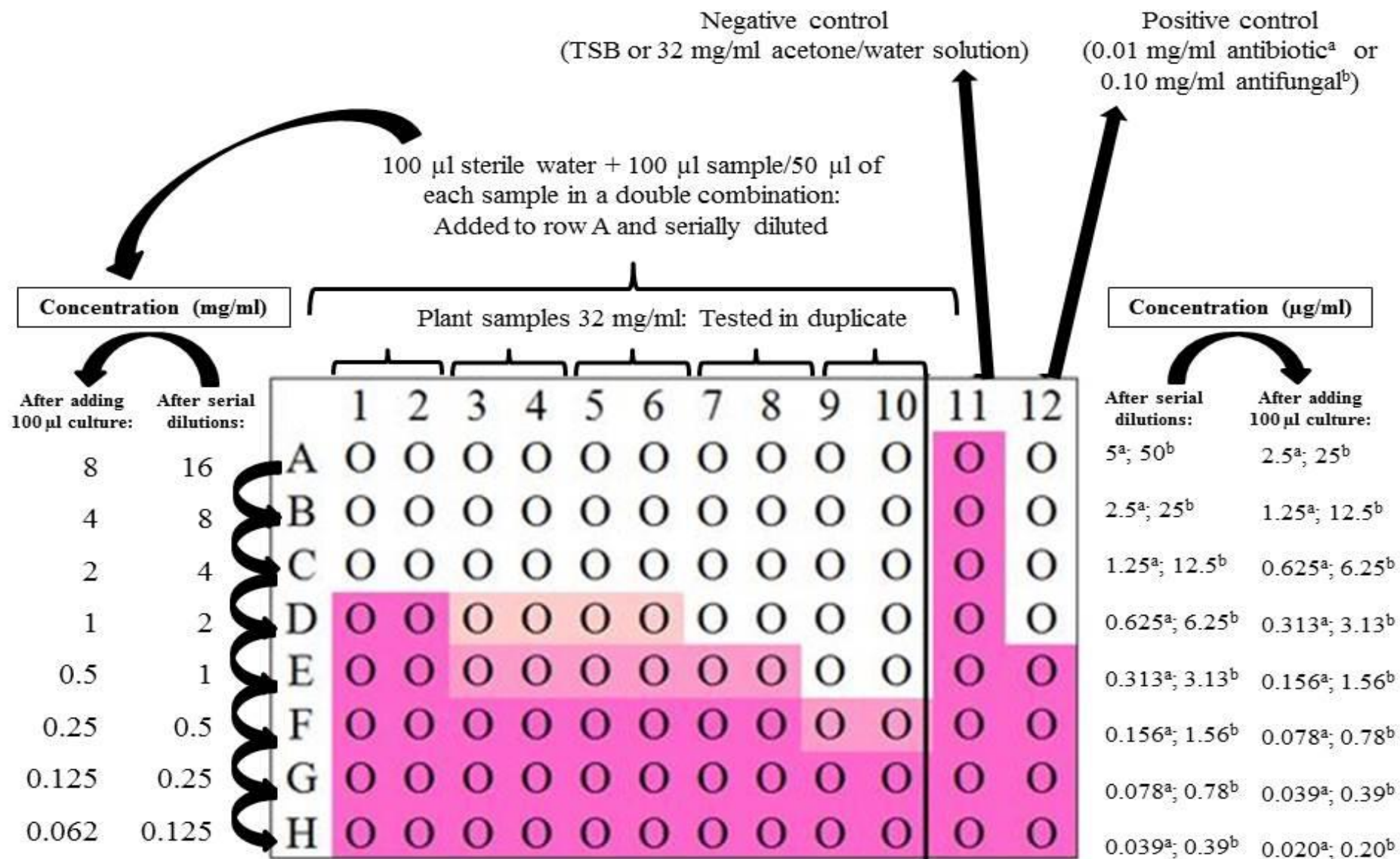


Figure 2.2. Graphical representation of an MIC plate, where the pink area indicates the wells with microbial growth. Reading of the MIC plate: Columns 1-6 have an MIC value of 2 mg/ml; Columns 7-8 an MIC value of 1 mg/ml; Columns 9-10 an MIC value of 0.5 mg/ml; Column 11 of TSB, no MIC is calculated; Column 12 of antimicrobial an MIC value of 0.313 µg/ml (antibiotic) or 3.13 µg/ml (antifungal).

Table 2.2. A summary of classifications for antimicrobial activity, according to MIC values.

Plant sample	MIC (mg/ml)	Classification of antimicrobial activity	Reference
Plant Extract	≥ 8	Very low antimicrobial activity	Fabry <i>et al.</i> , 1998
	< 8	Some antimicrobial activity	Fabry <i>et al.</i> , 1998; Padayachee, 2011; Suliman, 2011
	< 2	Potential antimicrobial activity	Duarte <i>et al.</i> , 2005
	≥ 1.6	Weak inhibition	Aligiannis <i>et al.</i> , 2001
	> 1	No antimicrobial activity	Rios and Recio, 2005
	< 1	Good antimicrobial activity	Ncube <i>et al.</i> , 2012
	< 1	Promising antimicrobial activity	McGaw <i>et al.</i> , 2007
	< 1	Noteworthy antimicrobial activity	Rios and Recio, 2005; Padayachee, 2011; Suliman, 2011; Van Vuuren, 2008
	0.6 – 1.5	Moderate inhibition	Aligiannis <i>et al.</i> , 2001
Essential oil	≤ 0.5	Strong inhibition	Aligiannis <i>et al.</i> , 2001
	≤ 2	Noteworthy antimicrobial activity	Duarte <i>et al.</i> , 2005; Van Vuuren, 2008; Padayachee, 2011; Suliman, 2011

Table 2.3. Classification of antimicrobial activity used in the current study.

Plant sample	MIC (mg/ml)	Classification of antimicrobial activity
Plant extract	< 1.00	Noteworthy
	1.00 – 3.00	Moderate
	$> 3.00 - < 8.00$	Weak or low
	≥ 8.00	Very low
Essential oil	≤ 2.00	Noteworthy

The relevant controls were included in each assay, which consisted of a positive control, along with three negative controls. The positive control was included in the assay to confirm antimicrobial susceptibility and consisted of:

- Ciprofloxacin (0.01 mg/ml) added as 100 μ l, when testing against bacteria, or
- Amphotericin B (0.1 mg/ml) added as 100 μ l, when testing against yeasts.

The two negative controls included in the plating process was a culture and solvent control:

- The culture control consisted of only TSB (100 μ l), along with the microbial culture (i.e. sample-free).
- The solvent control consisted of 100 μ l acetone/water solution (32 mg/ml), when testing organic extracts or essential oils.

The culture control was included in each plate to ascertain whether the media was capable of supporting microbial growth and also served as a means of determining when MIC results could be read. The culture control was absent of test sample, therefore the micro-organism would grow in all the wells, allowing for a purple-pink colour change in the presence of INT. Once the colour change was observed, the results for the entire plate could be read. The solvent control was included to ascertain whether the solvent exhibited any of its' own antimicrobial effects.

A further negative control, known as the media control was used, but not included in the plating process. The media control was used in order to determine whether the media used to support the growth of the micro-organisms in the micro-titre plate was sterile. The media control consisted of a sealed bottle containing some of the TSB media used in the plating process. The bottle of media was left out at room temperature overnight to check for the presence of any turbidity, which would indicate contamination of all the wells in the plate. If the TSB was found to be contaminated, the experiment would be repeated.

2.6. Fractional inhibitory concentration assessment

Interactions between the combinations of plant samples and conventional antimicrobials were further investigated using the sum of the fractional inhibitory concentration (Σ FIC) calculation, which would allow for the classification of the type of interaction occurring.

The MIC values obtained when the antimicrobial and plant sample when combined in equal volumes (50 μ l antimicrobial at a starting concentration of 0.1 mg/ml or 0.01 mg/ml and 50 μ l plant sample at a starting concentration of 32 mg/ml), were used for the Σ FIC calculation.

The Σ FIC was calculated using the following equation, where (a) represents the plant sample and (b) the conventional antimicrobial sample (Van Vuuren and Viljoen, 2011):

$$FIC^{(i)} = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}}$$

$$FIC^{(ii)} = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

The FIC index is then calculated using the following equation: $\Sigma FIC = FIC^{(i)} + FIC^{(ii)}$. Depending on the value obtained for the combination, the interactions could be classified as synergistic for a Σ FIC value of ≤ 0.5 , additive ($> 0.5 - 1.0$), non-interactive (indifferent) ($> 1.0 - \leq 4.0$) or antagonistic (> 4.0) (Van Vuuren and Viljoen, 2011).

Tentative interpretations were included, where the MIC value of one of the individual agents in the combination was greater than the highest concentration tested (> 8 mg/ml). Tentative interpretations provided an indication of the possible interactive profile for the combination; however, were not given an Σ FIC value since the calculation could only be undertaken on absolute values and ‘greater than’ values could not be considered in the calculation.

2.7. Varied ratio combination studies (isobolograms)

For notable interactions (synergistic or antagonistic interactions), nine different ratios of the combination were prepared and the MIC values determined. The samples were prepared at fixed concentrations of 0.01 or 0.1 mg/ml for antibiotic or antifungal respectively, and 32 mg/ml for the plant sample, as in the MIC analysis (Section 1.8.2). The relevant plants samples and antimicrobials were then combined in various volumes, which resulted in ratios of varying concentrations (Table 2.4). The combinations, prepared in various ratios, were then tested using the MIC assay.

Table 2.4. The concentration ratios used for antimicrobial and plant sample combination studies.

Volume ratio of antimicrobial: plant sample (μl)	Concentration of antibacterial ^a in combination (mg/ml)	Concentration of antifungal ^b in combination (mg/ml)	Concentration of plant sample in combination (mg/ml)
90:10	0.009	0.09	3.2
80:20	0.008	0.08	6.4
70:30	0.007	0.07	9.6
60:40	0.006	0.06	12.8
50:50	0.005	0.05	16.0
40:60	0.004	0.04	19.2
30:70	0.003	0.03	22.4
20:80	0.002	0.02	25.6
10:90	0.001	0.01	28.8

^a = ciprofloxacin / erythromycin / gentamicin / penicillin G / tetracycline; ^b = amphotericin B / nystatin

Data points for each ratio studied were plotted on an isobologram generated with GraphPad Prism[®] software (Version 5), after which the interactions for the various ratios of a combination could be classified as being synergistic, additive, non-interactive or antagonistic, based on the location of the data point on the isobologram (Figure 2.3). Data points falling below and on the 0.5:0.5 line (quadrant A) indicated synergy, while those above the 0.5:0.5 line, but below and including the 1.0:1.0 line (quadrant B) indicated an additive effect. Data points above the 1.0:1.0 line, but below and including the 4.0:4.0 line (quadrant C) indicated a non-interactive (indifferent) effect and those falling above the 4.0:4.0 line (quadrant D) indicated antagonism (Figure 2.3) (Van Vuuren and Viljoen, 2011). The construction of isobolograms allowed for the identification of the agent (plant or antimicrobial sample) most responsible for the synergistic or antagonistic effects within the combination.

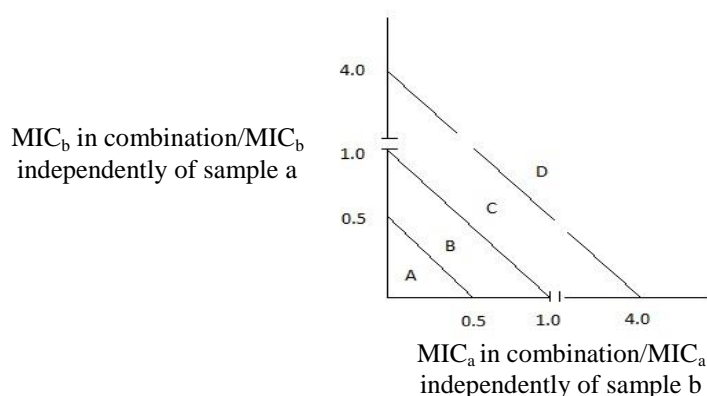


Figure 2.3. Isobologram used in the interpretation of a varied ratio combination study.

2.8. Toxicity studies

Two assays, namely the brine-shrimp lethality assay (BSLA) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay, were employed in order to determine possible toxicity of the plant samples and antimicrobials individually. Some combinations demonstrating notable interactions were also assessed for toxicity. The BSLA was used as a preliminary screening of the samples and combinations for possible toxicity. The MTT assay was used for a more in-depth cellular toxicological analysis.

Testing of samples for toxicity on a cell line also served as a means of eliminating the possibility of a false-positive in the antimicrobial analysis, allowing for the differentiation between an antimicrobial effect and a toxic effect (Cos *et al.*, 2006). Performing both toxicity assays allowed for a comparison, to determine whether toxicity results were consistent over both assays.

2.8.1. Brine-shrimp lethality assay

The BSLA is a rapid bioassay which allows for the screening of toxicity of natural products (McLaughlin *et al.*, 1998). This simple tool was used to screen for toxicity of all the plant samples and antimicrobial agents, independently as well as in combination. Methods followed were those proposed by Michael *et al.* (1956), which were later modified by Vanhaecke *et al.* (1981) and Sleet and Brendel (1983).

The BSLA was carried out using *Artemia franciscana* (Figure 2.4). In the presence of a toxic sample, the brine-shrimp would cease to survive, which would be observed by an absence of internal or external movement during a period of ten seconds (Carballo *et al.*, 2002). During the assay, brine-shrimp are not fed, as they are able to survive for 48 hours on the nutrients from the yolk-sac (Figure 2.4.c) (Lewis, 1995; Pelka *et al.*, 2000).

2.8.1.1. Brine-shrimp egg hatching

Artificial salt water was prepared by dissolving 32 g of Tropic Marine[®] sea salt in one litre of distilled water. An inverted, bottomless, transparent plastic bottle was filled with 500 ml of prepared artificial salt water, to which 0.5 g of dried, brine-shrimp (*A. franciscana*) eggs (Ocean Nutrition[™]) were added. To ensure a high hatch rate, a rotary pump (Kiho) was used for the aeration of salt water and the dispersion of eggs; and the eggs exposed to constant light with the use of a lamp. The eggs were incubated at 25 °C for 18 – 24 hours.



Figure 2.4. *Artemia franciscana* nauplius when viewed under an inverted microscope (Nikon) (a = hatching of brine-shrimp at 40x magnification; b = brine-shrimp after 24 hours at 100x magnification, demonstrating the yolk sac; c = brine-shrimp after 48 hours at 100x magnification).

2.8.1.2. Sample preparation

All samples were tested for toxicity at a concentration of 1 mg/ml. If higher concentrations (above 1 mg/ml) were necessary to exhibit a toxic effect against the brine-shrimp, then the sample could not be considered toxic in nature (Bussmann *et al.*, 2011). Therefore, for this study, samples were screened at a maximum concentration of 1 mg/ml. Samples (plant or antimicrobial), were prepared to a concentration of 2 mg/ml, to take into account the dilution factor of two when plating out. Where possible, samples were dissolved using distilled water. In the case where samples were insoluble in water, an organic solvent, such as DMSO, was used to dissolve the samples. The concentration of solvent used was always kept below 1%, to ensure that the solvent itself did not exert any toxic effect. Samples to be tested in combination were prepared in the same manner, except to a concentration of 4 mg/ml, to take into account the dilution factor of four when plating out.

2.8.1.3. Method for the brine-shrimp lethality assay

The artificial salt water containing the brine-shrimp was transferred to a shallow rectangular container, which was placed at an angle, and exposed to a concentrated light source for 30 minutes, which attracted the brine-shrimp, allowing for the withdrawal of high quantities of brine-shrimp. A volume of 400 μ l salt water containing on average 40 – 60 brine-shrimp, was withdrawn and added to each well of a 48 well micro-titre plate. For the testing of individual samples, 400 μ l plant sample or antimicrobial were added to a well, with samples being tested in triplicate per plate and repeated in two independent experiments, so that samples are tested in six separate wells ($n = 6$). For the screening of combinations, 200 μ l plant samples

and 200 µl antimicrobial sample was added to a well and tested in triplicate per plate, with at least two independent experiments undertaken (n = 6).

The negative, toxic-free control consisted of 32 g/l salt water and was used to mimic the natural environment of the brine-shrimp, thereby supporting their survival and growth. The positive control consisted of 1.6 mg/ml potassium dichromate (Sigma-Aldrich), which would ensure the mortality of all brine-shrimp present in the well, as it is known to be a highly toxic compound. Quinine (Sigma-Aldrich) and camptothecin (Sigma-Aldrich) were also screened for toxicity, at a concentration of 1 mg/ml, for comparison with the MTT assay, where these two agents were used as a positive control.

The plates were observed under a light microscope (Olympus) (magnification of 40x) immediately after sample addition (at time 0) for any dead brine-shrimp, which would be excluded from the total mortality calculation. Dead brine-shrimp were then counted after 24 and 48 hours of exposure to the test samples. After the dead brine-shrimp count at 48 hours, a lethal dose of 50 µl of glacial acetic acid (100% v/v, Saarchem) was added to each well for a total dead brine-shrimp count to be undertaken, such as to calculate the percentage mortality. Samples that induced a percentage mortality greater than 50% were considered biologically toxic (Bussmann *et al.*, 2011). These samples were then tested at concentrations of 1, 0.5, 0.25, 0.125, 0.063 and 0.031 mg/ml to generate a log-sigmoid dose response curve, using GraphPad Prism[®] software (Version 5), from which the LC₅₀ value was determined. The LC₅₀ value represents the concentration of a test substance necessary to have a lethal effect on 50% of the brine-shrimp. In the current study, LC₅₀ values below 249 µg/ml were considered highly toxic, 250 – 499 µg/ml considered as moderate in toxicity, 500 – 999 µg/ml were considered weak or low in toxicity and LC₅₀ values ≥ 1000 were considered non-toxic (Bussmann *et al.*, 2011).

2.8.2. The tetrazolium cellular viability assay

The MTT assay was used to determine the toxicity profile of the extracts and essential oils studied. The human kidney epithelial cell line, also known as the Graham or HEK-293 cell line, was used for this toxicity assay. A waiver for the use of the human kidney epithelial (Graham) cell line was granted by the WITS Human Research Ethics Committee (Reference W-CJ-120309-3) (Appendix I).

2.8.2.1. Cell culturing

The human kidney epithelial cells were cultured in media consisting of 13.5 g/l of Dulbecco's Modified Eagles Medium (DMEM) (Sigma-Aldrich) and 1.5 g/l of sodium bicarbonate (Sigma-Aldrich), dissolved in sterile Milli-Q[®] (double distilled) water. The media was sterilized through 0.2 µm filters (Masterflex) and placed in an incubator at 37 °C for four days to check for any turbidity, which would indicate contamination. Once sterility had been confirmed, the media was stored at 4 °C until needed. Before use, the media would be allowed to warm to ambient temperature before coming into contact with the cells. Before addition to the cells, the DMEM was supplemented with 10% (v/v) foetal bovine serum (FBS) (Thermo Scientific), 1% (v/v) non-essential amino acids (Sigma-Aldrich) and 1% (v/v) penicillin/streptomycin/fungizone mixture, consisting of 10,000 units penicillin/ml, 10,000 µg streptomycin/ml and 25 µg fungizone/ml (Sigma- Aldrich), providing a solution known as complete culture media. The FBS was inactivated prior to use, by thawing it out in the refrigerator at 4 °C and then allowing it to warm to room temperature, after which it was placed in a water bath at 56 °C for 30 minutes.

The cells were maintained at 37 °C in an incubator (Thermo Scientific) providing 5% carbon dioxide gas (CO₂), in accordance with methods by Mosmann (1983) and Van Zyl *et al.* (2006). The culture media was replaced every 48 hours, until confluency was achieved.

2.8.2.2. Trypsinisation of cells

Once confluency of the cells had been achieved, the spent media was discarded and the flasks washed with phosphate buffered saline (PBS). The PBS (pH 7.2) consisting of 8 g/l sodium chloride (Saarchem), 0.3 g/l potassium chloride (Sigma-Aldrich), 0.73 g/l sodium phosphate dibasic dihydrate (Reidel-de-Häen) and 0.2 g/l potassium phosphate monobasic (Fluka) was autoclave sterilized. Trypsin-EDTA solution (4 ml; Sigma-Aldrich) was added to each flask and left for three minutes at room temperature. Complete culture media (6 ml) was then added to the flasks to deactivate the trypsin. The cell suspension was centrifuged (Hettich Zentrifugen) at 1500 rpm for five minutes. Thereafter, the cells were re-suspended in fresh culture media (2 ml complete culture media for re-seeding of culture or 10 ml experimental culture media for use in an MTT assay) (Section 2.8.2.3). Experimental culture media was prepared by adding 10% (v/v) FBS to the DMEM. The non-essential amino acids and penicillin/streptomycin/fungizone mixture were not included in this media.

2.8.2.3. Method for the tetrazolium cellular viability assay

A volume of 50 µl of the re-suspended trypsinised cells was added to 50 µl of 0.2% (w/v) Trypan blue (Sigma-Aldrich), to create a 1:1 ratio. This solution was then used to count the number of cells/ml at a 100x magnification (Nikon), using a haemocytometer (Marienfeld). The cell suspension (> 95% cell viability) was then adjusted with experimental culture media, to ensure a concentration of 0.5 million cells/ml. The adjusted cell suspension (180 µl) was then added to each well of a sterile, 96 well micro-titre plate (VWR International). To ensure that the cells had adhered to the well surface, the plates were incubated at 37 °C for 6 hours, in a humidified environment, with 5% CO₂ before the addition of test sample.

Stock solutions of extracts and essential oils, all prepared to a concentration of 10 mg/ml in DMSO, were then serially diluted to obtain the required starting concentration, taking into account the dilution factors. Plant extracts and essential oils were screened at 100 µg/ml. Samples were further tested at a concentration of 1 mg/ml for comparison with the BSLA. However, the colour of the plant samples often resulted in numerous washing steps to prevent interference of sample colour with the absorbance readings. Excessive washing lead to compromised cells; therefore these results were not taken into account. The samples were then further tested at a concentration of 500 µg/ml, however, cells were compromised again, and thus results were inaccurate and the data were not taken into account for the toxicity analysis of samples in this study.

The appropriately diluted samples (20 µl of extract and 2 µl of essential oil) were then plated out. Each sample was tested in triplicate per plate, along with a colour control (absence of cell suspension) for each sample, which consisted of 180 µl experimental culture media and a total volume of 20 µl of sample when testing plants extracts or antimicrobials, or 2 µl essential oil with 18 µl of culture media. Included in each plate were two wells of a cell-free, sample-free control, consisting only of 200 µl experimental culture media; three wells of the positive control, quinine (Sigma-Aldrich) or camptothecin (Sigma-Aldrich), for comparison to the untreated wells of 100% cell suspension control (Figure 2.5). The two positive controls have been described in detail in Appendix J. The plates were then incubated at 37 °C for a further 44 hours in the same humidified conditions.

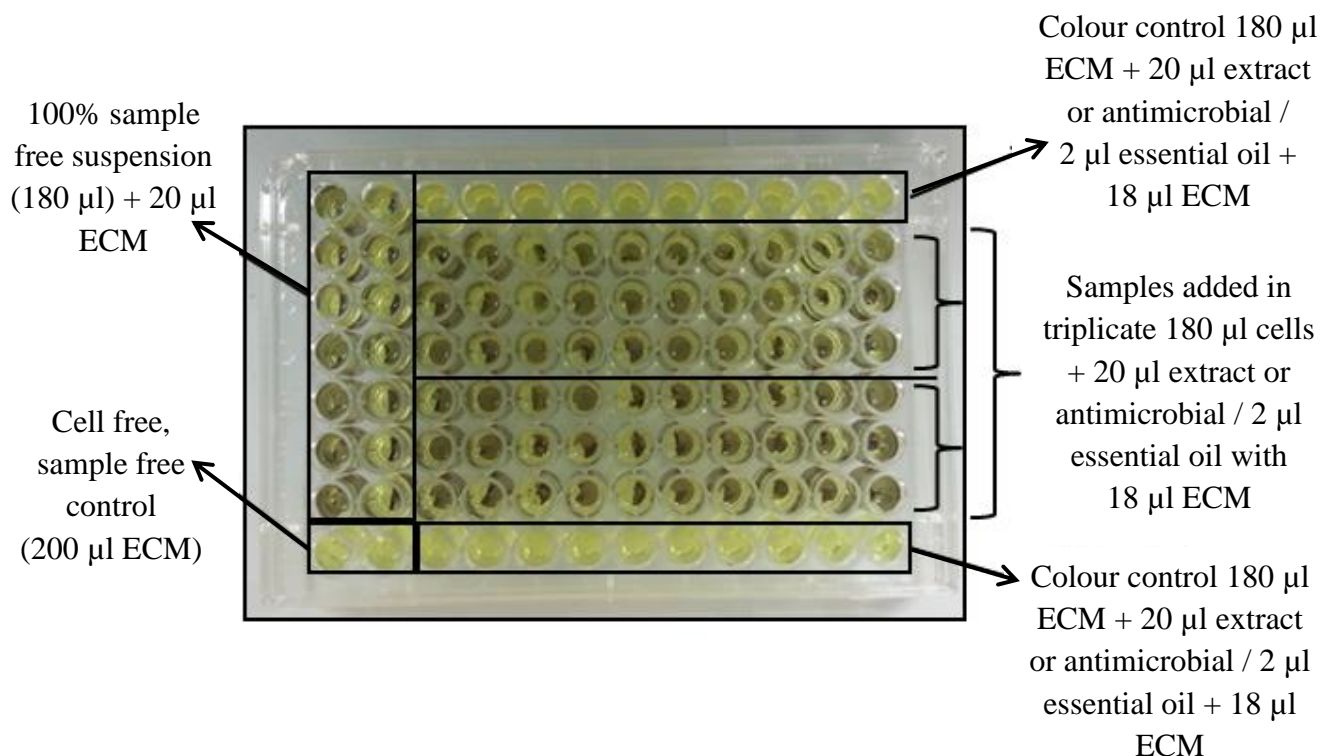


Figure 2.5. Plating out for a MTT assay. ECM = experimental culture media.

A washing step was then undertaken, by removing 150 µl of supernatant and replacing it with PBS. This process was undertaken three times, or a maximum of five times, depending on the colour of the sample. The washing step was to ensure no interference of plant sample colour in the absorbance readings and to prevent any potential interaction with of the samples with the MTT. Thereafter, 40 µl sterile filtered MTT (5.00 mg/ml; Sigma-Aldrich) solution prepared in PBS, was added to each well and the plates re-incubated for a further four hours.

Thereafter, a volume of 170 µl of the supernatant was then removed from each well, ensuring no disruption of the adherent cells, and replaced with DMSO (Rochelle Chemicals), to stop the reaction and to solubilise the purple formazan crystals, which formed in the presence of active mitochondria of viable cells (Figure 2.6).

The absorbance of the dissolved crystals were read at a test wavelength of 540 nm and reference wavelength of 690 nm, using the Labsystems iEMS MF reader connected to a computer with Ascent[®] software. The absorbance values of the formazan product reflected the amount of live cells present, since a linear relationship exists between absorbance and live cells (Mosmann, 1983).

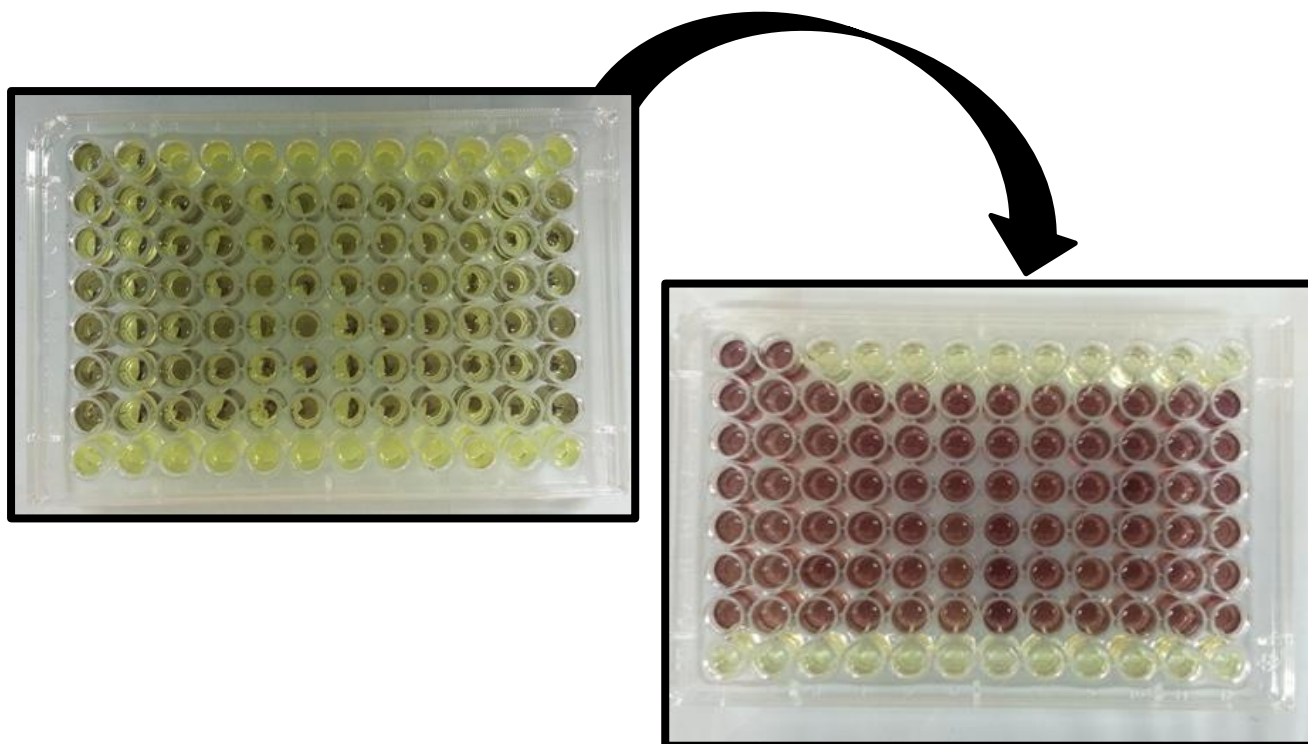


Figure 2.6. The colour change which occurred once the formazan crystals were dissolved in DMSO.

The results were then expressed as a percentage cell viability, using the following equation:

$$\% \text{ Cell death} = 100 - \left[\frac{\text{Abs test sample} - (\text{Mean Abs control} - \text{Mean Abs blank})}{(\text{Mean Abs control} - \text{Mean Abs blank})} \right] \times 100$$

Where “Abs” represents absorbance. All absorbance values used in the calculation were derived from deducting the reference absorbance value at 690 nm from the test absorbance value at 540 nm ($\text{Abs}_{540} - \text{Abs}_{690}$) (Kamatou, 2006).

Samples, individually or in combination, showing a toxic effect were then further evaluated at various concentrations to generate a log-sigmoid dose response curve, using GraphPad Prism[®] software (Version 5) for the determination of an IC_{50} value. The IC_{50} value represents the concentration of test substance necessary to cause the inhibition of 50% of cells. A one in two dilution was used, such that at least five dilutions were used for the construction of a log-sigmoid dose response curve. A percentage cellular viability less than 50% was considered toxic in this study, when testing samples at a concentration of 100 $\mu\text{g/ml}$ (Naidoo, 2013).

Chapter 3

The antimicrobial activity of plant samples and conventional antimicrobials when tested individually

3.1. Introduction

Medicinal plants have been used for centuries as a primary source for fighting infections and this long history of use provides some support for the antimicrobial activity of these medicinal plants (Rabe and Van Staden, 1997). However, in order for the medicinal plants to be accepted into conventional healthcare, as an alternative to conventional drugs, scientific data is necessary to support the traditional claims that have been made. Some of the medicinal plants selected for analysis in this study have been claimed to be effective in the traditional treatment of some infections, particularly those of the respiratory tract and of the skin. However, some are known to have very weak or no antimicrobial activity. The plants were still included in this study based on their commercial relevance and popularity in traditional healing practices, which would contribute to the possibility of concurrent use with conventional drugs. In order to determine the interactive antimicrobial profiles when the selected medicinal plants and conventional antimicrobials are combined, these agents needed to be assessed individually for antimicrobial activity. Therefore, in this chapter, the aim of the study was to firstly validate the susceptibility of the selected micro-organisms towards the conventional antimicrobials to be studied. The antimicrobial efficacy of the conventional antimicrobials have already been well characterised. However, for this study, it was necessary to test the antimicrobial activity of the conventional antimicrobials individually, to obtain the necessary data for determining the interactive effects of the selection of antimicrobials with the medicinal plants. The aim of this chapter was also to ascertain whether the tested conventional antimicrobials had MIC values that fell within the expected breakpoint ranges, which was needed to guarantee the accuracy and reliability of results. Lastly, the antimicrobial activity of the independent plant samples had to be assessed for antimicrobial activity, to obtain the necessary data for the combination studies.

3.2. Results and discussion

3.2.1. Antimicrobial activity of conventional antimicrobials

The MIC values obtained for the individual antimicrobial agents when tested against the selected pathogens have been recorded in Table 3.1. Results obtained in this study were compared with breakpoint expectations (Table 3.2) derived from previous antimicrobial studies, which are a range of expected MIC values of an antimicrobial when tested against a specific micro-organism. Ciprofloxacin was most effective against *E. coli* (MIC of 0.08 µg/ml) and erythromycin against *S. aureus* and *B. cereus*, equally (MIC of 0.32 µg/ml). Gentamicin was most effective against the Gram-negative micro-organism, *P. aeruginosa* (MIC of 0.32 µg/ml). Penicillin G, showed equal efficacy against all three tested Gram-positive pathogens (MIC of ≥ 2.50 µg/ml) and tetracycline was most active against *B. cereus* (MIC of 0.16 µg/ml). The antifungals, amphotericin B and nystatin, were both found to be more active against *C. neoformans* (MIC of 0.39 µg/ml and 1.56 µg/ml, respectively) than against *C. albicans* (Table 3.1).

Table 3.1. MIC values (µg/ml) for all conventional antimicrobial agents, when tested individually.

	Antibiotics					Antifungals	
	Cip	Ery	Gen	Pen	Tet	Amp	Nys
<i>S. aureus</i> (ATCC 25923)	0.47	0.32	1.88	≥ 2.50	0.23	NS	NS
<i>B. cereus</i> (ATCC 11778)	0.63	0.32	≥ 2.50	≥ 2.50	0.16	NS	NS
<i>E. faecalis</i> (ATCC 29212)	1.25	1.25	≥ 2.50	≥ 2.50	≥ 2.50	NS	NS
<i>E. coli</i> (ATCC 25922)	0.08	NS	≥ 2.50	NS	1.25	NS	NS
<i>K. pneumoniae</i> (ATCC 13883)	0.63	NS	≥ 2.50	NS	1.25	NS	NS
<i>P. aeruginosa</i> (ATCC 27853)	0.16	NS	0.32	NS	≥ 2.50	NS	NS
<i>C. albicans</i> (ATCC 10231)	NS	NS	NS	NS	NS	1.56	2.34
<i>C. neoformans</i> (ATCC 14116)	NS	NS	NS	NS	NS	0.39	1.56

Cip = ciprofloxacin; Ery = erythromycin; Gen = gentamicin; Pen = penicillin G; Tet = tetracycline; Amp = amphotericin B; Nys = nystatin; NS = micro-organism is not susceptible to the antimicrobial; ≥ 2.50 = antimicrobial samples were not tested at higher concentrations for the determination of a MIC value.

Table 3.2. Breakpoint ranges for commercial antimicrobial agents (MIC values in µg/ml).

	<i>S. aureus</i> (ATCC 25923)	<i>B. cereus</i> (ATCC 11778)	<i>E. faecalis</i> (ATCC 29212)	<i>E. coli</i> (ATCC 25922)	<i>K. pneumoniae</i> (ATCC 13883)	<i>P. aeruginosa</i> (ATCC 27853)	<i>C. albicans</i> (ATCC 10231)	<i>C. neoformans</i> (ATCC 14116)
Ciprofloxacin	a. 0.12 – 0.5 b. 0.06 – 128 c. 0.5 – ? d. 0.12 – 0.50	a. – b. – c. 0.2 – ? d. 0.20 – 1.00	a. 0.25 – 2 b. 0.25 – 128 c. 1 – ? d. 0.25 – 2.00	a. 0.004 – 0.015 b. 0.004 – 128 c. 0.004 – 0.016 d. 0.004 – 0.016	a. – b. 0.004 – 128 c. – d. 0.12 – 0.80	a. 0.25 – 1 b. 0.015 – 128 c. 0.6 – ?	NS	NS
Erythromycin	a. 0.25 – 1 b. 0.06 – 128 c. 0.25 – ?	a. – b. – c. –	a. 1 – 4 b. 0.25 – 128 c. 1 – ?	a. – b. – c. –	a. – b. – c. –	a. – b. – c. –	NS	NS
Gentamicin	a. 0.12 – 1 b. 0.008 – 128 c. 0.12 – ?	a. – b. – c. 3.12 – ?	a. 4 – 16 b. 0.5 – 2048 c. ? – 12.5	a. 0.25 – 1 b. 0.03 – 128 c. 0.12 – 2	a. – b. 0.03 – 128 c. –	a. 0.5 – 2 b. 0.06 – 128 c. –	NS	NS
Penicillin G	a. 0.25 – 2 b. 0.015 – 128 c. 0.015 – ?	a. – b. – c. –	a. 1 – 4 b. 0.5 – 128 c. 2 – ?	a. – b. – c. > 8 – ?	a. – b. – c. –	a. – b. – c. –	NS	NS
Tetracycline	a. 0.12 – 1 b. 0.06 – 128 c. 0.125 – ?	a. – b. – c. –	a. 8 – 32 b. – c. 16 – ?	a. 0.5 – 2 b. 0.25 – 128 c. 0.2 – ?	a. – b. 0.25 – 128 c. –	a. 8 – 32 b. – c. – d. – e. 4000	NS	NS
Amphotericin B	NS	NS	NS	NS	NS	NS	a. – b. – c. 0.125 – ? d. 1.25 – 2.50 e. – f. 0.06 – 2	a. – b. – c. 39 – ?
Nystatin	NS	NS	NS	NS	NS	NS	a. – b. – c. 0.49 – ?	a. – b. – c. –

a. = CLSI guidelines, 2012; b. = Andrews, 2004; c. = KnowledgeBase antimicrobial index; d. = Van Vuuren, 2007; e. NCCLS guidelines, 1997; f. = O'Shaughnessy, 2009; NS = micro-organism not susceptible to antimicrobial; ? = end point is unspecified; – = no MIC breakpoint values available.

3.2.1.1. Ciprofloxacin

Ciprofloxacin is a broad-spectrum antimicrobial agent, expected to have activity against both Gram-negative and Gram-positive bacteria, and shows no activity toward yeasts (Merck Manual, 2006; SAMF, 2012). Against the selected pathogens, the antimicrobial efficacy of ciprofloxacin was expected to be between 0.004 µg/ml and 128 µg/ml, with respect to the breakpoint ranges. Results obtained in this study for ciprofloxacin, show MIC values between 0.08 µg/ml (lowest MIC value and found against *E. coli*) and 1.25 µg/ml (highest MIC value and found against *E. faecalis*) (Table 3.1). Ciprofloxacin, prepared at a concentration of 0.01 mg/ml, exhibited MIC values in this study, that were all within the breakpoint ranges against all the tested pathogens.

3.2.1.2. Erythromycin

Erythromycin is a narrow-spectrum antimicrobial agent, with activity expected mostly against Gram-positive bacteria (Merck Manual, 2006; SAMF, 2012). Erythromycin, when tested against the appropriate pathogens, was expected to have MIC values between 0.06 µg/ml and 128 µg/ml, according to breakpoint ranges. In this study, erythromycin showed MIC values between 0.32 µg/ml (lowest MIC value and found against *S. aureus* and *B. cereus*) and 1.25 µg/ml (highest MIC value and found against *E. faecalis*) (Table 3.1). Therefore, when prepared at a concentration of 0.01 mg/ml, erythromycin demonstrated MIC values that were well within the breakpoint ranges against all the tested pathogens.

3.2.1.3. Gentamicin

Gentamicin is a narrow-spectrum antimicrobial agent, with activity expected mostly against Gram-negative micro-organisms, but can show some Gram-positive activity (Merck Manual, 2006; SAMF, 2012). Gentamicin, when prepared at 0.01 mg/ml and tested against the appropriate pathogens and was expected to have antimicrobial efficacy ranging between 0.008 µg/ml and 2048 µg/ml. Results obtained in this study show MIC values for gentamicin between 0.32 µg/ml (lowest MIC value and against *P. aeruginosa*) and values greater than 2.50 µg/ml (highest MIC value and against *B. cereus* and *E. faecalis*), between all the tested pathogens (Table 3.1). When looking at the activity against the individual pathogens, MIC values for gentamicin were all well within the breakpoint ranges.

3.2.1.4. Penicillin G

Penicillin G is a narrow-spectrum antibiotic, with activity focused on Gram-positive micro-organisms (Merck Manual, 2006; SAMF, 2012). The antimicrobial efficacy for penicillin G was expected at breakpoint ranges between 0.015 µg/ml and 128 µg/ml. When penicillin G was prepared at a concentration of 0.01 mg/ml, an MIC value of 2.50 µg/ml was obtained against the tested pathogens (Table 3.1). When evaluating the activity of penicillin G against the individual tested pathogens, the MIC values were all found to be within the breakpoint ranges for each particular pathogen.

3.2.1.5. Tetracycline

Tetracycline is a broad-spectrum antibiotic, demonstrating activity against both Gram-positive and Gram-negative micro-organisms (Merck Manual, 2006; SAMF, 2012). Tetracycline was expected to show antimicrobial efficacy between 0.06 µg/ml and 4000 µg/ml, according to breakpoint ranges (Table 3.2). The MIC values for tetracycline, obtained in this study, ranged from 0.16 µg/ml (lowest MIC values and against *B. cereus*) to 2.50 µg/ml (highest MIC value and against *E. faecalis* and *P. aeruginosa*) (Table 3.1). When evaluating the activity of tetracycline against the appropriate pathogens, the MIC values were mostly found to be within the breakpoint ranges for each particular pathogen, except where tetracycline was tested against *E. faecalis* and *P. aeruginosa* (Table 3.1). The breakpoint expectation against *E. faecalis* ranges between 8 µg/ml and 32 µg/ml (Table 3.2) and the MIC value obtained in this study against *E. faecalis* was 2.50 µg/ml (Table 3.1), which is below the expected range. The breakpoint expectation against *P. aeruginosa* ranges between 8 µg/ml and 4000 µg/ml, and the MIC value obtained in this study against *P. aeruginosa* was 2.50 µg/ml (Table 3.1), which again, is below the expected range. Tetracycline was tested again, in triplicate, to verify the MIC values against *E. faecalis* and *P. aeruginosa* and the same MIC values were obtained with each repeat experiment. The lower MIC values against tetracycline, in comparison to the breakpoint expectations could be attributed to an unusual susceptibility of the *E. faecalis* and *P. aeruginosa* strains used in this study, which would not be of concern in the study, since MIC values below the breakpoint still demonstrate sensitivity of the pathogen towards the antimicrobial, as opposed to being above the breakpoint, which would indicate resistance of strains towards the antimicrobial (Levison, 2004).

3.2.1.6. Amphotericin B

Amphotericin B is an antifungal agent, with activity against candidal and cryptococcal species (Merck Manual, 2006; SAMF, 2012). The antimicrobial efficacy of this agent is expected between ranges of 0.06 µg/ml and 39.00 µg/ml (Table 3.2). The MIC values obtained for amphotericin B in this study (Table 3.1), when prepared at 0.10 mg/ml, ranged between 0.39 µg/ml (lowest MIC value against *C. albicans*) and 1.56 µg/ml (highest MIC value against *C. neoformans*). Breakpoint ranges for antifungal agents are not readily available, however, the MIC value of amphotericin B against *C. albicans* was found to be within the available breakpoint range. Breakpoint ranges for *C. neoformans* could not be taken into account, due to the lack of references available regarding breakpoint ranges against *C. neoformans*.

3.2.1.7. Nystatin

Nystatin is an antifungal agent, with activity against candidal and cryptococcal species (Merck Manual, 2006; SAMF, 2012). The breakpoint ranges for this agent were not readily available, therefore, could not be compared to breakpoints. However, nystatin was tested in triplicate on separate days, in order to ensure accuracy of obtained MIC values and demonstrated the same MIC value with each repetition, when prepared at a concentration of 0.10 mg/ml. Results obtained in this study provided a mean MIC value of 2.34 µg/ml against *C. albicans* and 1.56 µg/ml against *C. neoformans* (Table 3.1).

3.2.2. Antimicrobial activity of medicinal plants

The medicinal plants selected for the study were not necessarily selected on the basis of their use as an antimicrobial, but rather that these plants are amongst those classified as the most popularly used medicinal plants in South Africa. The antimicrobial activities of all the selected plants have already been examined to some extent (Appendix F). For this study, the plant samples were still assessed for antimicrobial activity, in order to provide the necessary data required for the combination studies. The MIC results for the plant samples were recorded graphically (Figure 3.1 for the aqueous extracts, Figure 3.2 for the organic extracts and Figure 3.3 for the essential oils) and summarised in Table 3.3. In general, most of the plants tested demonstrated poor antimicrobial activity, which was expected. A few exceptions have, however, been noted where noteworthy antimicrobial activity was identified. The medicinal plants that showed the most promising antimicrobial activity was *A. betulina*,

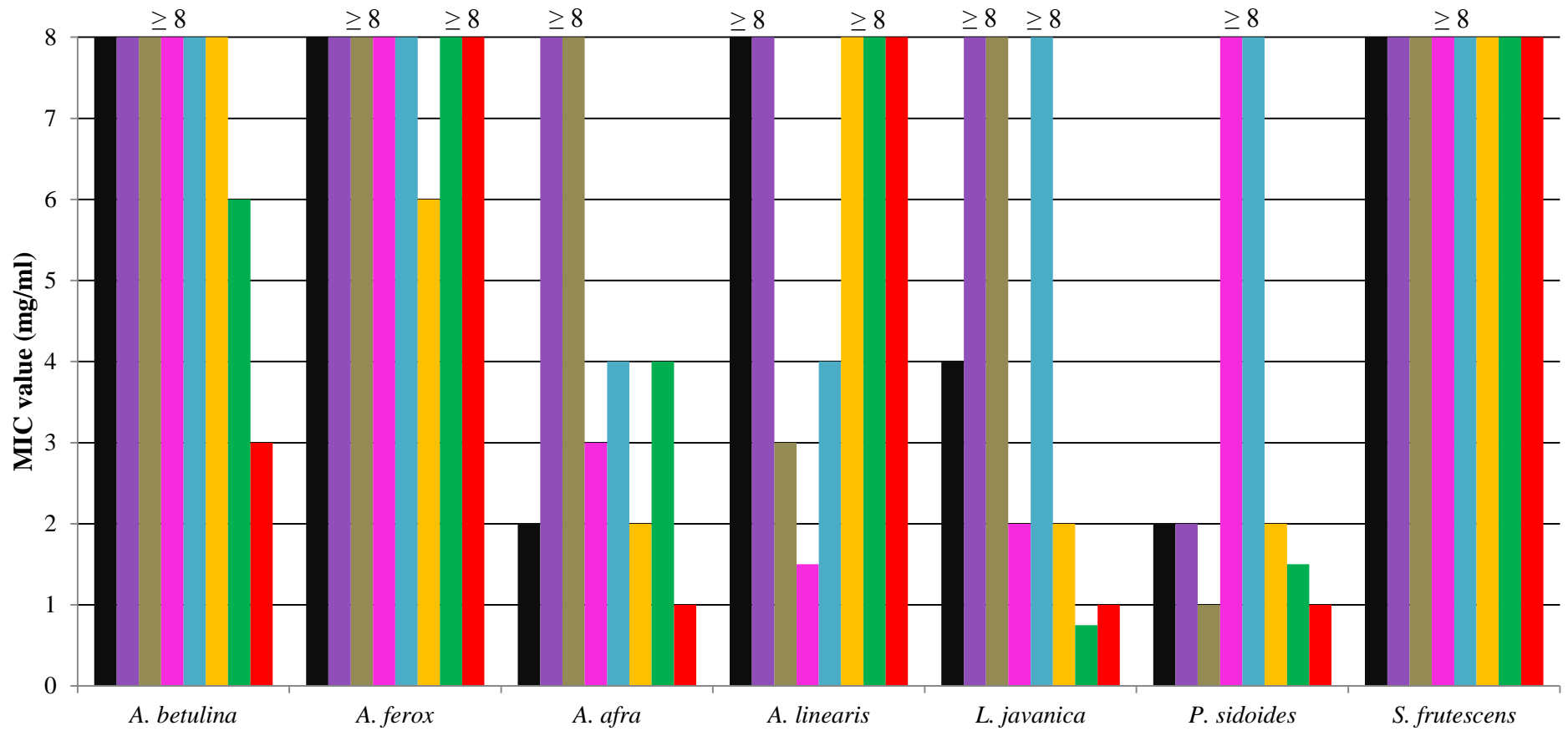


Figure 3.1. The aqueous extracts of all the selected plants and their MIC values against the tested pathogens (■ = *S. aureus*; ■ = *B. cereus*; ■ = *E. faecalis*; ■ = *E. coli*; ■ = *K. pneumoniae*; ■ = *P. aeruginosa*; ■ = *C. albicans*; ■ = *C. neoformans*).

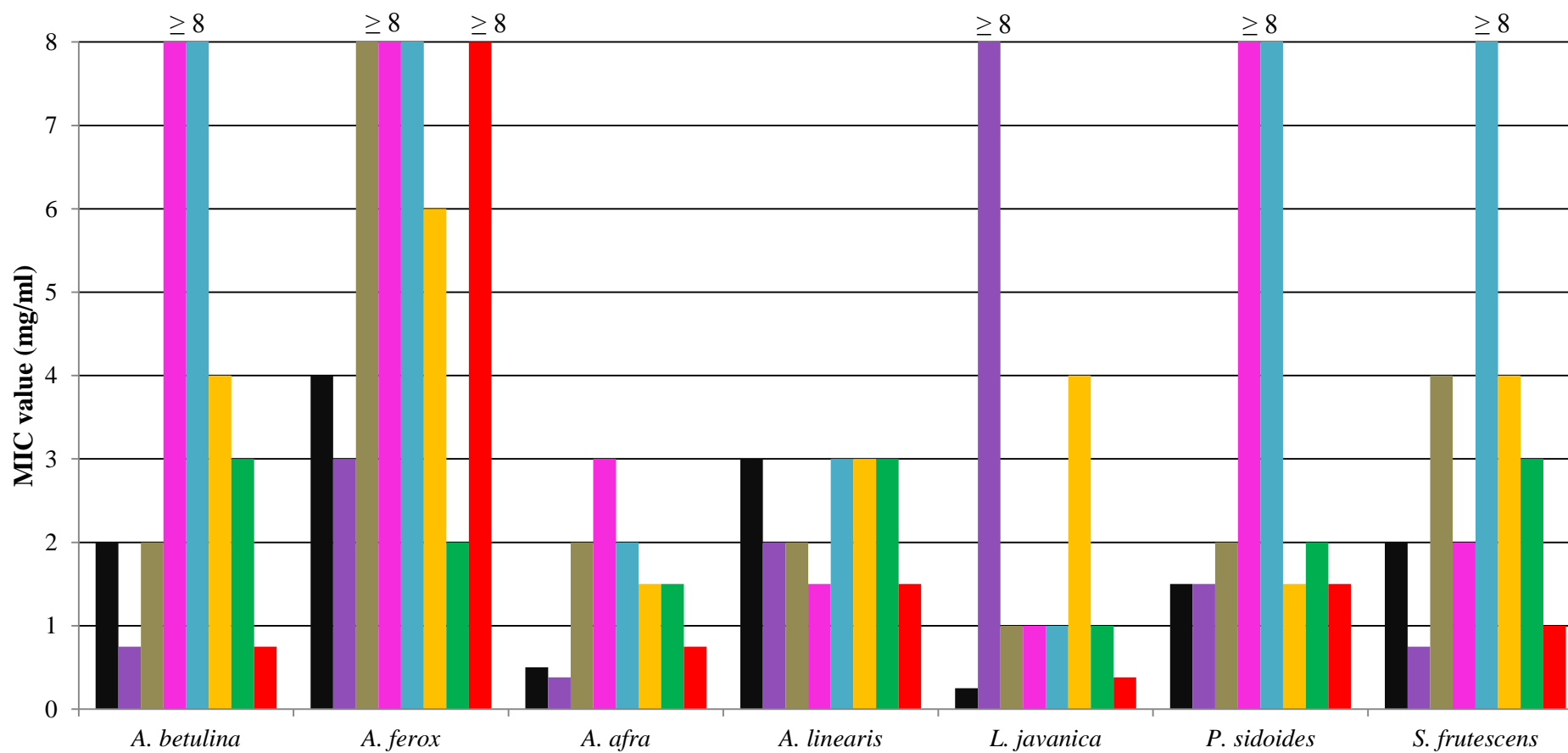


Figure 3.2. The organic extracts of all the selected plants and their MIC values against the tested pathogens (■ = *S. aureus*; ■ = *B. cereus*; ■ = *E. faecalis*; ■ = *E. coli*; ■ = *K. pneumoniae*; ■ = *P. aeruginosa*; ■ = *C. albicans*; ■ = *C. neoformans*).

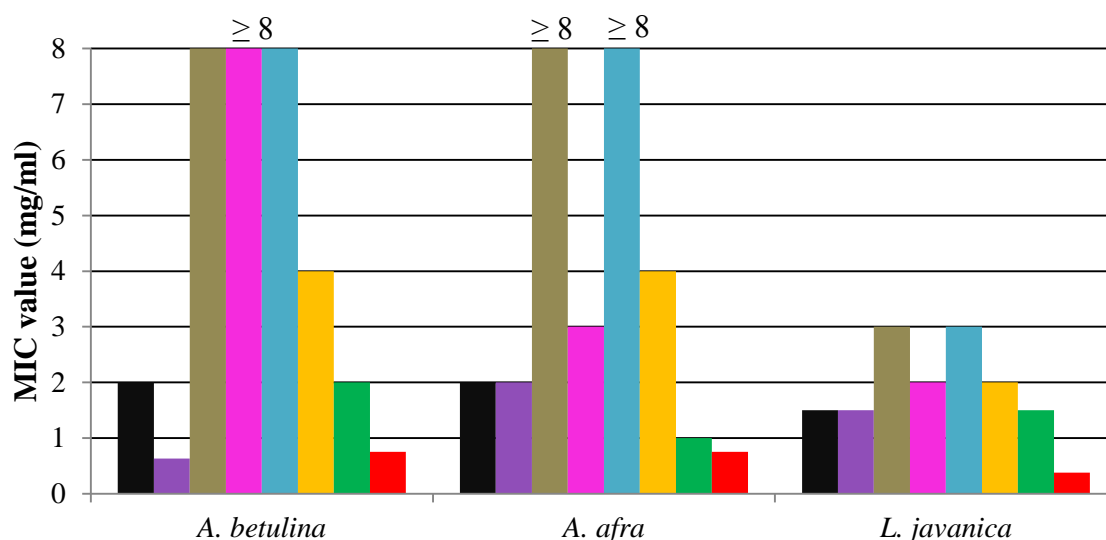


Figure 3.3. The essential oils of the aromatic plants and their MIC values against the tested pathogens (■ = *S. aureus*; ■ = *B. cereus*; ■ = *E. faecalis*; ■ = *E. coli*; ■ = *K. pneumoniae*; ■ = *P. aeruginosa*; ■ = *C. albicans*; ■ = *C. neoformans*).

A. afra and *L. javanica*, where the organic extracts and essential oils mostly provided noteworthy activity, as opposed to the aqueous extracts. It was evident from the MIC values that the conventional antimicrobials (Table 3.1) exhibited a far greater antimicrobial activity than the medicinal plants (Table 3.3).

For the aqueous extracts (Figure 3.1), *A. afra*, *L. javanica* and *P. sidoides* were the only plants to demonstrate noteworthy antimicrobial activity, where most often, the noteworthy activity was seen against the yeasts (Figure 3.1). It is apparent from Figure 3.1 that overall, *A. betulina*, *A. ferox* and *S. frutescens* aqueous extracts demonstrated the least broad-spectrum antimicrobial activity against all the tested pathogens, whereas *A. afra*, *A. linearis*, *L. javanica* and *P. sidoides* demonstrated more broad-spectrum activity (Figure 3.1). The aqueous extract of *P. sidoides* demonstrated the broadest spectrum of activity, with the lowest mean MIC value of 3.19 mg/ml (Table 3.3). However, the aqueous extract of *L. javanica* displayed the lowest MIC value (strongest antimicrobial activity) among the aqueous plant extracts tested, which was seen against *C. albicans* (MIC of 0.75 mg/ml) (Table 3.3) and was the only noteworthy antimicrobial activity demonstrated by an aqueous extract. The aqueous extract of *S. frutescens* demonstrated the least antimicrobial activity across the eight tested pathogens, with the highest mean MIC value of 8.00 mg/ml (Table 3.3). It has been stated

Table 3.3. Summary of the MIC values (mg/ml) for all plant samples, when tested individually.

	<i>S. aureus</i> (ATCC 25923)			<i>B. cereus</i> (ATCC 11778)			<i>E. faecalis</i> (ATCC 29212)			<i>E. coli</i> (ATCC 25922)			<i>K. pneumoniae</i> (ATCC 13883)			<i>P. aeruginosa</i> (ATCC 27853)			<i>C. albicans</i> (ATCC 10231)			<i>C. neoformans</i> (ATCC 14116)			Mean MIC*		
	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO	Aq	Org	EO
<i>A. betulina</i>	≥8.00	2.00	2.00	≥8.00	0.75	0.63	≥8.00	2.00	≥8.00	≥8.00	≥8.00	≥8.00	≥8.00	≥8.00	≥8.00	≥8.00	4.00	4.00	6.00	3.00	2.00	3.00	0.75	0.75	7.13	3.56	4.17
<i>A. ferox</i>	≥8.00	4.00	NA	≥8.00	3.00	NA	≥8.00	≥8.00	NA	≥8.00	≥8.00	NA	≥8.00	≥8.00	NA	6.00	6.00	NA	≥8.00	2.00	NA	≥8.00	≥8.00	NA	7.75	4.88	NA
<i>A. afra</i>	2.00	0.50	2.00	≥8.00	0.38	2.00	≥8.00	2.00	≥8.00	3.00	3.00	3.00	4.00	2.00	≥8.00	2.00	1.50	4.00	4.00	1.50	1.00	1.00	0.75	0.75	4.00	1.45	3.59
<i>A. linearis</i>	≥8.00	3.00	NA	≥8.00	2.00	NA	3.00	2.00	NA	1.50	1.50	NA	4.00	3.00	NA	≥8.00	3.00	NA	≥8.00	3.00	NA	≥8.00	1.50	NA	6.06	2.38	NA
<i>L. javanica</i>	4.00	0.25	1.50	≥8.00	≥8.00	1.50	≥8.00	1.00	3.00	2.00	1.00	2.00	≥8.00	1.00	3.00	2.00	4.00	2.00	0.75	1.00	1.50	1.00	0.38	0.38	4.22	2.08	1.86
<i>P. sidoides</i>	2.00	1.50	NA	2.00	1.50	NA	1.00	2.00	NA	≥8.00	≥8.00	NA	≥8.00	≥8.00	NA	2.00	1.50	NA	1.50	2.00	NA	1.00	1.50	NA	3.19	3.25	NA
<i>S. frutescens</i>	≥8.00	2.00	NA	≥8.00	0.75	NA	≥8.00	4.00	NA	≥8.00	2.00	NA	≥8.00	≥8.00	NA	≥8.00	4.00	NA	>8.00	3.00	NA	≥8.00	1.00	NA	8.00	3.09	NA
Cip control	0.00047			0.00063			0.00125			0.00008			0.00063			0.00016			NS			NS			–		
Amp control	NS			NS			NS			NS			NS			NS			0.00156			0.00039			–		
S/M control	≥8.00			≥8.00			≥8.00			≥8.00			≥8.00			≥8.00			≥8.00			≥8.00			–		

All values of ≥ 8.00 mg/ml were taken as 8.00 mg/ml for the calculation of the mean MIC value for each plant; Aq = Aqueous extract; Org = Organic extract; EO = Essential oil; NA = Not applicable as no/insufficient essential oil evident in the species or part of plant tested; NS = control not tested, since the particular micro-organism is not susceptible to the antimicrobial; **bold highlight** = noteworthy antimicrobial activity; Cip = ciprofloxacin; Amp = amphotericin B; S/M = solvent or media control.

that aqueous extracts of plants usually show little or no antimicrobial activity (Meyer and Afolayan, 1995; Masika and Afolayan, 2002), hence it is not surprising to find poor activity for the aqueous extracts of the plants studied here. The inclusion of aqueous extracts, even with their poor activity, was important in this study, since medicinal plants are most commonly prepared for consumption using water. Therefore, the aqueous extract mimics the traditional use of the medicinal plant most accurately.

For the organic extracts (Figure 3.2), it is apparent that *A. ferox* demonstrated the least broad-spectrum antimicrobial activity, with the highest mean MIC value of 4.88 mg/ml (Table 3.3). Noteworthy antimicrobial activity was seen for the organic extracts of *A. betulina*, *A. afra*, *L. javanica* and *S. frutescens* (Table 3.3). The organic extracts of *A. afra*, *L. javanica* and *P. sidoides* demonstrated the most broad-spectrum activity, with *A. afra* demonstrating the broadest spectrum of activity (lowest mean MIC value of 1.45 mg/ml). The organic extract of *A. linearis* also showed some promising broad-spectrum activity. As with the aqueous extracts, the organic extract of *L. javanica* again, displayed the lowest MIC value (strongest antimicrobial activity) among the selected organic plant extracts; however, it is against *S. aureus* and not *C. albicans*, as seen with the aqueous extract of *L. javanica*. The yeasts (*C. albicans* and *C. neoformans*), as well as most of the Gram-positive micro-organisms showed the most susceptibility towards the organic extracts of the medicinal plants, whereas the Gram-negative pathogens, particularly *E. coli* and *K. pneumoniae*, tended to be less susceptible to the organic extracts (Figure 3.2).

The essential oils for the three aromatic plants (*A. betulina*, *A. afra* and *L. javanica*) were also tested for antimicrobial activity and it was apparent (Figure 3.3), that the essential oil of *L. javanica* definitely showed the broadest spectrum of antimicrobial activity against all the tested pathogens, with the lowest mean MIC value of 1.86 mg/ml (Table 3.3). All three essential oils tested did, however, show some noteworthy antimicrobial activity. Again, it was found that *L. javanica* showed the lowest MIC value (strongest antimicrobial activity), when the essential oil was tested against the yeast, *C. neoformans* (Table 3.3). Generally for the three essential oils, the least activity was seen against *E. faecalis* and *K. pneumoniae*. *Agathosma betulina* and *A. afra* essential oils were very similar in activity, except against *E. coli*, where *A. afra* had a greater activity than *A. betulina* essential oil (Figure 3.3).

When testing the antimicrobial activity of plant samples in the micro-dilution assay, various controls were included. The MIC values obtained for the positive controls of ciprofloxacin

for bacteria and amphotericin B for yeasts (Table 3.3) were compared with the breakpoint ranges (Table 3.2) and were found to be within these ranges. The MIC values obtained for the controls are included in Table 3.3, but have been left out of the bar graphs in Figure 3.1, Figure 3.2 and Figure 3.3, due to the differential scales.

3.2.2.1. *Agathosma betulina*

In this study, the aqueous extract of *A. betulina* demonstrated no noteworthy antimicrobial activity against all the eight tested pathogens. The best antimicrobial activity exhibited by the aqueous extract of *A. betulina* was against the yeast, *C. neoformans* (MIC of 3.00 mg/ml) (Table 3.3), which was considered moderate in antimicrobial activity for this study (Table 2.3).

The organic extract of *A. betulina* showed noteworthy antimicrobial activity against the Gram-positive micro-organism, *B. cereus* (MIC of 0.75 mg/ml) and the yeast, *C. neoformans* (MIC of 0.75 mg/ml) (Table 3.3). In a study by Moolla (2005), an organic extract (methanol: dichloromethane, 1:1), prepared from the leaves of *A. betulina* was tested against *B. cereus* (MIC of 4.00 mg/ml), *S. aureus* (MIC of 4.00 mg/ml), *K. pneumoniae* (MIC of 4.00 mg/ml) and *C. albicans* (2.00 mg/ml), using the micro-dilution assay. No noteworthy antimicrobial activity was found. This is congruent with the current study, except when the organic extract was tested against *B. cereus*, where a noteworthy MIC value of 0.75 mg/ml was found in this study (Table 3.3). The strains of *B. cereus* and *C. albicans* used were the same as in this current study, whereas the strains of *S. aureus* and *K. pneumoniae* differed, which could contribute towards the varied MIC values obtained.

The essential oil of *A. betulina*, when tested in this study, showed noteworthy antimicrobial activity (MIC of ≤ 2.00 mg/ml) against four of the eight tested pathogens, namely the Gram-positive micro-organisms, *S. aureus* (MIC of 2.00 mg/ml) and *B. cereus*, having the most antimicrobial activity (MIC of 0.63 mg/ml), along with both the yeasts tested, *C. albicans* (MIC of 2.00 mg/ml) and *C. neoformans* (MIC of 0.75 mg/ml) (Table 3.3). Lis-Balchin *et al.* (2001) tested the antimicrobial activity of the essential oil (10 μ l of undiluted oil) of *A. betulina*, against *S. aureus*, *E. coli* and *P. aeruginosa*, using the agar diffusion assay. The antimicrobial activity was classified as being virtually non-existent against *P. aeruginosa* (4.00 mm zone of inhibition) and very low against *S. aureus* (5.80 mm zone of inhibition) and *E. coli* (6.00 mm zone of inhibition). These antimicrobial classifications against *S. aureus* and *P. aeruginosa* were not supported by the current study (Table 3.3). In contrast,

noteworthy antimicrobial activity (MIC of ≤ 2.00 mg/ml) was seen against *S. aureus* and against *P. aeruginosa* (MIC of 4.00 mg/ml) the antimicrobial activity was classified as being low or weak (Table 2.3), rather than very low, as seen in the study by Lis-Balchin *et al.* (2001). The variation in results between this current study and results found by Lis-Balchin *et al.* (2001) could be attributed to the different testing techniques undertaken. It has been acknowledged that many problems arise when testing antimicrobial activity of essential oils using the diffusion method. This is due to the lipophilic nature of the oil samples, which makes diffusion through the agar difficult, resulting in a false negative (Hewitt and Vincent, 2003; Van Vuuren, 2008). Evaporation of the essential oil from the discs upon incubation can also result in a false negative, particularly with the yeasts, where incubation periods are longer (Janssen *et al.*, 1987). The classification of “very low” antimicrobial activity against *E. coli* by Lis-Balchin *et al.* (2001) was in congruence with the current study, where an MIC of greater than 8.00 mg/ml was obtained, demonstrating very low antimicrobial activity for this study. Moolla (2005) tested the essential oil of *A. betulina* against *B. cereus* (MIC of 4.00 mg/ml), *S. aureus* (MIC of 4.00 mg/ml), *K. pneumoniae* (MIC of 4.00 mg/ml) and *C. albicans* (2.00 mg/ml), using the micro-dilution assay. Noteworthy antimicrobial activity (MIC of ≤ 2.00 mg/ml) was also found against *C. albicans* (MIC of 2.00 mg/ml). The findings by Moolla (2005) regarding *C. albicans* are in accordance with results from this study. However, the findings by Moolla (2005) concerning *S. aureus*, *B. cereus* and *K. pneumoniae* are not similar to those found in the current study (Table 3.3), since noteworthy antimicrobial activity was seen against *B. cereus* (MIC of 0.63 mg/ml) and *S. aureus* (2.00 mg/ml) and the MIC obtained against *K. pneumoniae* was ≥ 8.00 mg/ml, indicating very low antimicrobial activity (Table 2.3). This variance could be due to the different strains of *S. aureus* and *K. pneumoniae* that were used in each study.

3.2.2.2. *Aloe ferox*

In the current study, the aqueous extract of *A. ferox* demonstrated no noteworthy antimicrobial activity against the tested pathogens (Table 3.3). The extract was most active against *P. aeruginosa* (MIC of 6.00 mg/ml) (Table 3.3), which was considered as weak or low antimicrobial activity in this study (Table 2.3). A study by Van Vuuren and Naidoo (2010) investigated the antimicrobial activity of *A. ferox* aqueous extract, prepared from the leaves of the plant, against *C. albicans*, using the micro-dilution assay. An MIC value of 4.00 mg/ml was obtained, thereby demonstrating no noteworthy antimicrobial activity against *C. albicans*. The lack of noteworthy antimicrobial activity was also evident in the current study

(Table 3.3). Kambizi *et al.* (2007) also found no antimicrobial activity of the aqueous extract of *A. ferox* against *C. albicans*.

The organic extract, when tested in this current study, exhibited no noteworthy antimicrobial activity against any of the tested pathogens (Table 3.3). It was most active against the yeast, *C. albicans* (MIC of 2.00 mg/ml), which was considered moderate in antimicrobial activity (Table 2.3). Kambizi *et al.* (2007) found that a methanol extract of *A. ferox* was not active against *C. albicans*. Van Vuuren and Naidoo (2010) investigated the organic extract (dichloromethane: methanol; 1:1) of *A. ferox* against *C. albicans* (MIC of 3.00 mg/ml) and found that again, no noteworthy antimicrobial activity was exhibited, as seen with the aqueous extract of *A. ferox*.

3.2.2.3. *Artemisia afra*

In the current study, the aqueous extract of *A. afra* demonstrated no MIC values below 1.00 mg/ml, therefore, no noteworthy antimicrobial activity was identified (Table 3.3). The aqueous extract of *A. afra* was found to be most active against the yeast, *C. neoformans* (MIC of 1.00 mg/ml) (Table 3.3), where moderate antimicrobial activity was exhibited (Table 2.3). In a study by McGaw *et al.* (2000), aqueous extracts of *A. afra* were tested against *S. aureus*, *K. pneumoniae* and *E. coli*, using the micro-dilution assays. No noteworthy antimicrobial activity was identified against these pathogens, which is in accordance with the current study.

The organic extract, when tested in the current study, demonstrated noteworthy antimicrobial activity against *S. aureus* (MIC of 0.50 mg/ml), *B. cereus* (0.38 mg/ml) and *C. neoformans* (MIC of 0.75 mg/ml) (Table 3.3). The organic extract was therefore found to be most active against the Gram-positive micro-organism, *B. cereus* (MIC of 0.38 mg/ml) (Table 3.3). Against the remaining tested pathogens, the organic extract showed MIC values between 1.50 mg/ml and 3.00 mg/ml, which were considered moderate in activity (Table 2.3). In a study by McGaw *et al.* (2000), organic extracts (hexane and ethanol extracts) of *A. afra* were tested against *S. aureus*, *K. pneumoniae* and *E. coli*, using the micro-dilution assay. The hexane extract demonstrated no noteworthy antimicrobial activity, whilst the ethanol extract had an MIC value of 0.39 mg/ml against *S. aureus*, thereby demonstrating noteworthy antimicrobial activity. No noteworthy antimicrobial activity was found against the other two pathogens. In the current study, noteworthy activity was also found against *S. aureus* (MIC of 0.50 mg/ml) for the organic extract (dichloromethane: methanol, 1:1) (Table 3.3). The variance in MIC

values could be due to the different solvent systems used for extraction purposes (ethanol versus dichloromethane: methanol).

In the current study, the essential oil of *A. afra* exhibited noteworthy antimicrobial activity (MIC of ≤ 2.00 mg/ml), against four of the eight tested pathogens, namely *S. aureus* and *B. cereus* (MIC of 2.00 mg/ml), *C. albicans* (MIC of 1.00 mg/ml) and *C. neoformans* (MIC of 0.75 mg/ml) (Table 3.3). The essential oil was therefore found to be most active against *C. neoformans* (MIC of 0.75 mg/ml). Suliman *et al.* (2010) investigated the essential oil of *A. afra*, against *K. pneumoniae*, *E. faecalis* and *C. neoformans*, using the micro-dilution assay. No noteworthy antimicrobial activity was identified for the essential oil against these three tested pathogens in the investigation, as the MIC values ranged from 6.00 mg/ml to 16.00 mg/ml. These findings by Suliman *et al.* (2010) are congruent with the current study findings, except against *C. neoformans*, where in this study the essential oil did in fact demonstrate noteworthy antimicrobial activity. Huffman *et al.* (2002) tested the essential oil of *A. afra* against *S. aureus* (minimum inhibitory percentage (MIP) of $> 1\%$), *P. aeruginosa* (MIP of $> 9\%$), *C. albicans* (MIP of 0.25%) and *C. neoformans* (MIP of 0.5%), using the micro-dilution assay. Van Vuuren and Viljoen (2006) tested the antimicrobial activity of *A. afra* essential oil on *S. aureus*, *B. cereus*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *C. albicans* and *C. neoformans*, using the micro-dilution assay. According to the classifications used in the current study (Table 2.3), no noteworthy antimicrobial activity was identified for the essential oil, since MIC values ranged from 4.50 mg/ml to 11.90 mg/ml. These results are incongruent with the findings in the current study (Table 3.3), where the essential oil of *A. afra* demonstrated noteworthy antimicrobial activity against four of the pathogens. The variation in results is most likely due to inter-population variation in the essential oil composition, which has been noted in a study by Viljoen *et al.* (2006).

3.2.2.4. *Aspalathus linearis*

In the current study, the aqueous extract of *A. linearis* did not demonstrate any noteworthy antimicrobial activity (MIC < 1.00 mg/ml) against any of the eight tested pathogens (Table 3.3). The lack of noteworthy antimicrobial is expected since *A. linearis* is more well-known for its antimutagenic and anti-oxidant activity, rather than for its antimicrobial activity (Van Wyk *et al.*, 2009). Many studies have investigated the antimicrobial activity of green and black teas (Toda *et al.*, 1989; Diker *et al.*, 1991; Fukai *et al.*, 1991; Diker and Hascelik, 1994; Yeo *et al.*, 1995), however, *A. linearis* has not been extensively investigated. Two studies

found on the antimicrobial activity of *A. linearis* were conducted by Schepers (2001) and Coetzee *et al.* (2008). Schepers (2001) identified the inhibitory effects of the soluble solids of unfermented and fermented aqueous extract of *A. linearis* at concentrations varying from 0.50 g/l to 5.00 g/l, against a range of pathogens. The growth of the pathogens when exposed to the extracts was determined spectrophotometrically, to obtain optical densities for broths containing culture and sample. It was found that rooibos had an inhibitory effect on *S. aureus*, *B. cereus* and *E. coli* after 12 hours of exposure. The growth profiles, when the aqueous extract was tested at 5.00 mg/ml, demonstrated a reduced final cell concentration (N_{max}) and maximum specific growth rate (μ_{max}). The growth of *S. aureus* was reduced by 90.80% (fermented) and 50.10% (unfermented) and the growth of *B. cereus* was reduced by 80.30% (fermented) and 47.2% (unfermented). The growth of *E. coli* was reduced by 69.00% (fermented) and 35.10% (unfermented). The strongest inhibitory effect in the study by Schepers (2001) was found against *S. aureus*, which is incongruent with findings in the current study, since it was found that the aqueous rooibos extract displayed the best antimicrobial activity against *E. coli* (MIC value of 1.50 mg/ml) (Table 3.3). Coetzee *et al.* (2008) also identified the inhibitory effects of aqueous rooibos extracts against *E. coli*, but the extracts were tested at very high concentrations and the inhibitory effect only seen at 10 mg/ml, therefore cannot be considered noteworthy in antimicrobial activity.

The organic extract of *A. linearis* tested in the current study exhibited no noteworthy antimicrobial activity against any of the tested pathogens (Table 3.3). The organic extract had the lowest MIC value against *E. coli* (MIC of 1.50 mg/ml), which were similar to the aqueous extract, and also against *C. neoformans* (MIC of 1.50 mg/ml), which are considered moderate in activity (Table 2.3). The results obtained in this study, where the organic extract (dichloromethane: methanol, 1:1) demonstrated somewhat better antimicrobial activity towards all tested pathogens than the aqueous extract (Table 3.3) are incongruent with findings by Schepers (2001). It was reported that the aqueous extract was more potent than the ethyl acetate extract. This variation may be due to the use of different solvents (ethyl acetate versus dichloromethane: methanol) for the preparation of the organic extract. Different solvent systems extract different compounds, resulting in the different activities. Schepers (2001) identified that rooibos organic extract, prepared using ethyl acetate, had an inhibitory effect against *E. coli* at 5 mg/ml, during growth profile studies. The growth of *E. coli* was reduced by 42.60% (fermented) and 5.20% (unfermented) by the ethyl acetate extract of rooibos. The inhibitory effects of the organic extract on *E. coli* was also seen in the

current study (Table 3.3), where an MIC of 1.50 mg/ml was obtained against *E. coli*, when a dichloromethane: methanol extract was tested.

3.2.2.5. *Lippia javanica*

In the current study, *L. javanica* aqueous extract demonstrated the most noteworthy antimicrobial activity against *C. albicans* (MIC of 0.75 mg/ml) (Table 3.3). It was also found to have moderate activity (Table 2.3) against the other tested yeast species, *C. neoformans* (MIC of 1.00 mg/ml) (Table 3.3).

The organic extract of *L. javanica* showed noteworthy antimicrobial activity against *S. aureus* (MIC of 0.25 mg/ml) and against the yeast, *C. neoformans* (MIC of 0.38 mg/ml) (Table 3.3). The organic extract was found to be most active against *S. aureus* (MIC of 0.25 mg/ml). Moderate antimicrobial activity (MIC of 1.00 mg/ml) was seen against four of the remaining tested pathogens, namely *E. faecalis*, *E. coli*, *K. pneumoniae* and *C. albicans*. Shikanga *et al.* (2010) found that the methanolic extracts of *L. javanica* showed noteworthy activity against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa*, during a micro-dilution assay, with MIC ranges between 0.13 mg/ml and 0.42 mg/ml. These findings are only congruent with the current study with regards to *S. aureus*. Variations in results could be due to varied strains of micro-organisms that were used and also different solvent use for extraction purposes (methanol versus dichloromethane: methanol in this study).

The essential oil of *L. javanica* demonstrated noteworthy antimicrobial activity, against six of the eight tested pathogens (Table 3.3), namely *S. aureus* (MIC of 1.50 mg/ml), *B. cereus* (MIC of 1.50 mg/ml), *E. coli* (MIC of 2.00 mg/ml), *P. aeruginosa* (MIC of 2.00 mg/ml), *C. albicans* (MIC of 1.50 mg/ml) and *C. neoformans* (MIC of 0.38 mg/ml). Huffman *et al.* (2002) found that *L. javanica* essential oil also demonstrated activity against *S. aureus* (MIP of 1%), *C. albicans* (MIP of 0.25%) and *C. neoformans* (MIP of 0.25%), however, not against *P. aeruginosa* (MIP of > 9%). Van Vuuren and Viljoen (2006) tested the essential oil of *L. javanica* against *S. aureus*, *B. cereus*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *C. albicans* and *C. neoformans*, and found mostly moderate to poor antimicrobial activity against the pathogens. Noteworthy antimicrobial activity was only seen against *E. coli* (MIC of 1.60 mg/ml), which was also observed in the current study, with an MIC of 2.00 mg/ml (Table 3.3).

3.2.2.6. *Pelargonium sidoides*

In the current study, the aqueous extract of *P. sidoides* demonstrated no noteworthy antimicrobial activity (Table 3.3). Moderate antimicrobial activity (Table 2.3) was, however, seen against *S. aureus*, *B. cereus* and *P. aeruginosa* (MIC of 2.00 mg/ml), along with *E. faecalis* and *C. neoformans* (MIC of 1.00 mg/ml) and *C. albicans* (MIC of 1.50 mg/ml). Kayser and Kolodziej (1997), tested the aqueous extract of *P. sidoides* against three Gram-positive bacteria, including *S. aureus*, and five Gram-negative bacteria, including *E. coli*, *K. pneumoniae* and *P. aeruginosa*. MIC values were found to range from 0.6 mg/ml for the aqueous phases to 10 mg/ml for crude extracts.

The organic extract of *P. sidoides* exhibited no noteworthy antimicrobial activity against any of the tested pathogens in the current study (Table 3.3). However, moderate antimicrobial activity (Table 2.3) was seen against six of the tested pathogens, namely *S. aureus*, *B. cereus*, *E. faecalis*, *P. aeruginosa*, *C. albicans* and *C. neoformans*, with an MIC range of 1.50 mg/ml to 2.00 mg/ml (Table 3.3). Kolodziej (2011) tested organic extracts of the plant, which displayed MIC values ranging from 600 – 7500 µg/ml against *S. aureus*, *Streptococcus pneumoniae*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Haemophilus influenzae*. Lewu *et al.* (2006) investigated the antimicrobial activity of *P. sidoides* acetone and methanol extracts prepared from the roots collected from various locations in the Eastern Cape, against *B. cereus* (MIC of 2.50 – 5.00 mg/ml for acetone extract, MIC of 5.00 mg/ml for methanol extract) and *S. aureus* (MIC of 1.00 – 2.50 mg/ml for acetone extract, MIC of 2.50 – 7.50 mg/ml for methanol extract). In the current study, MIC values of the organic dichloromethane: methanol extract against *B. cereus* (MIC of 1.50 mg/ml) and *S. aureus* (MIC of 1.50 mg/ml) were most similar with the MIC values of the acetone extracts obtained in the latter study and not the methanol extracts. In the study by Lewu *et al.* (2006), the extracts were also tested against the Gram-negative, *E. coli*, *K. pneumoniae* and *P. aeruginosa* and no noteworthy antimicrobial activity was found. Similarly, in the current study, no noteworthy antimicrobial activity was seen against *E. coli* (MIC of ≥ 8.00 mg/ml) and *K. pneumoniae* (MIC of ≥ 8.00 mg/ml). However, inhibitory effects were seen against *P. aeruginosa* (MIC of 1.50 mg/ml) (Table 3.3).

3.2.2.7. *Sutherlandia frutescens*

In the current study, the aqueous extract of *S. frutescens* showed no noteworthy antimicrobial activity against any of the eight tested pathogens, with MIC values all ≥ 8.00 mg/ml (Table

3.3). In a study by Katerere and Eloff (2005), an aqueous extract of *S. frutescens* was tested against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa*, where an MIC of 10.00 mg/ml was reported against each pathogen. The lack of noteworthy antimicrobial activity in the study by Katerere and Eloff (2005) is in accordance with findings in the current study.

In the current study, the organic extract of *S. frutescens* showed noteworthy antimicrobial activity against *B. cereus* (MIC of 0.75 mg/ml), whilst against the other pathogens, MIC values ranged from 1.00 mg/ml (moderate antimicrobial activity) to ≥ 8.00 mg/ml (very low antimicrobial activity) (Table 3.3). In a study by Katerere and Eloff (2005), various organic extracts of *S. frutescens* were tested against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa*. A dichloromethane extract was tested and an MIC value of 2.50 mg/ml was observed against *S. aureus* and 5.00 mg/ml against *E. faecalis*, *E. coli* and *P. aeruginosa*. Very similar MIC values (MIC of 2.00 – 4.00 mg/ml) were obtained in the current study (Table 3.3).

3.3. Conclusions

- All antimicrobials tested were within breakpoint ranges; however, studies with tetracycline demonstrated an enhanced susceptibility against *E. faecalis* and *P. aeruginosa*.
- When comparing the aqueous extracts of all the selected plants, *A. afra* demonstrated the best antimicrobial activity, with the lowest mean MIC of 3.19 mg/ml.
- The aqueous extract of *S. frutescens* demonstrated the lowest spectrum of antimicrobial activity (highest mean MIC value of 8.00 mg/ml) against the eight tested pathogens, in comparison with all other plant samples.
- When comparing the organic extracts of all plant samples with one another, the organic extract of *A. ferox* exhibited the least activity against the tested pathogens, with the highest mean MIC value of 4.88 mg/ml.
- The organic extract of *A. afra* exhibited the broadest spectrum of antimicrobial activity (lowest mean MIC value of 1.45 mg/ml) across the eight tested pathogens.

- The organic extract of *L. javanica* demonstrated the MIC value most noteworthy in nature, amongst all the tested plants, with a MIC value of 0.25 mg/ml against *S. aureus*.
- When comparing the antimicrobial activity of the essential oils from the three aromatic medicinal plants, *L. javanica* demonstrated the broadest spectrum of antimicrobial activity (lowest mean MIC of 1.86 mg/ml), whereas *A. betulina* demonstrated the least (highest mean MIC of 4.17 mg/ml).

Chapter 4

Combination antimicrobial studies

4.1. Introduction

Pharmaceutical drug-drug interactions have been a well-known factor for years. These interactions have often been found to be fatal and are therefore taken very seriously in the pharmaceutical industry and pharmacy practice. All pharmaceutical reference books (SAMF, Merck Manual, along with many others) and even patient leaflets provide a list of the possible interactions that a drug may have with other pharmaceutical preparations. However, very rarely, are the interactions with natural products specified in these sources of information. The only natural products that are usually specified for their interactions in these books and leaflets are those with St. John's wort (*Hypericum perforatum*) and grapefruit juice, which are well-known for their induction of hepatic CYP450 enzymes (SAMF, 2012).

Proper consultation with a healthcare provider should be emphasized when planning to use natural products in combination with orthodox medicine. Healthcare providers are familiar with the common herb-drug interactions, such as that between warfarin and *Ginkgo biloba* (ginkgo), resulting in excessive bleeding or the interaction between selective-serotonin re-uptake inhibitors and St. John's wort, resulting in serotonin syndrome. Another common herb-drug interaction is that between corticosteroids and *Glycyrrhiza glabra* (liquorice), resulting in the potentiation of the corticosteroids (Street and Prinsloo, 2012). There are websites dedicated to herb-drug interactions, such as www.prescribguide.com. However, very little is known about the interactions between medicinal plants commonly used in South African traditional healing practices, and conventional drugs.

Therefore, in this chapter, the study aimed to determine the antimicrobial efficacy and interactive profile when a selection of well-known South African medicinal plants were combined with seven conventional antimicrobials.

4.2. Results and discussion

A total of 476 combinations were evaluated. These were made up of a combination of seven South African medicinal plants (essential oil, aqueous and organic extract) with seven conventional antimicrobials, which were then tested against eight different pathogens. In order to examine the vast amount of data in more detail, results have been presented for each medicinal plant separately against the Gram-positive bacteria, Gram-negative bacteria and then the yeasts (Tables 4.1 – 4.21). The results have also been summarized in a general discussion.

4.2.1. Combinations containing *Agathosma betulina*

The essential oil, aqueous and organic extracts of *A. betulina* in combination with the conventional antimicrobials and tested against the Gram-positive micro-organisms (Table 4.1), demonstrated mostly additive and indifferent interactive profiles. No antagonistic interactions were identified against the Gram-positive pathogens. Three combinations (*A. betulina* aqueous and organic extract with gentamicin; *A. betulina* essential oil with tetracycline) were found to be synergistic, when tested against *B. cereus* (Table 4.1).

When testing all the combinations containing *A. betulina*, against the Gram-negative pathogens (Table 4.2), a mostly additive and indifferent interactive profile was observed, with no antagonism noted. Seven synergistic interactions were identified (*A. betulina* organic extract and essential oil with ciprofloxacin against *E. coli*; *A. betulina* aqueous and organic extract with gentamicin against *K. pneumoniae*), with no synergy found against *P. aeruginosa* (Table 4.2).

The combination of *A. betulina* with ciprofloxacin provided a notable interactive profile, when tested against *E. coli*, which is most often the cause of urinary tract infections (UTI's), causing approximately 75% of community-acquired UTI's (Merck Manual, 2006). UTI's present with symptoms of urinary frequency, urgency, dysuria and lower abdominal and flank pain. Similarly, in traditional medicine, *A. betulina* is commonly used for the treatment of UTI's (Appendix F.1). In orthodox medicine, the fluoroquinolones, such as ciprofloxacin, have been the first line therapy for UTI's for many years (Appendix G.1; Merck Manual, 2006).

Table 4.1. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *A. betulina* with the various antibiotics, against the Gram-positive pathogens.

Combination	Sample type	<i>S. aureus</i> (ATCC 25923)				<i>B. cereus</i> (ATCC 11778)				<i>E. faecalis</i> (ATCC 29212)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. betulina</i> + ciprofloxacin	Aq Cip	≥ 8000 0.47	2000 0.63	1.59	IND	≥ 8000 0.63	1000 0.32	NA	ADD	≥ 8000 1.25	3000 0.94	NA	IND
	Org Cip	2000 0.47	2000 0.63	2.34	IND	750 0.63	500 0.16	0.92	ADD	2000 1.25	2000 0.63	1.50	IND
	EO Cip	2000 0.47	1000 0.32	1.18	IND	630 0.63	1000 0.32	2.10	IND	≥ 8000 1.25	1500 0.47	NA	IND
<i>A. betulina</i> + erythromycin	Aq Ery	≥ 8000 0.32	1000 0.32	1.16	IND	≥ 8000 0.32	1000 0.32	NA	IND	≥ 8000 1.25	3000 0.94	NA	IND
	Org Ery	2000 0.32	2000 0.63	3.03	IND	750 0.32	500 0.16	1.19	IND	2000 1.25	≥ 4000 ≥ 1.25	NA	IND
	EO Ery	2000 0.32	1000 0.32	1.53	IND	630 0.32	380 0.12	0.99	ADD	≥ 8000 1.25	2000 0.63	NA	ADD
<i>A. betulina</i> + gentamicin	Aq Gen	≥ 8000 1.88	≥ 4000 ≥ 1.25	1.17	IND	≥ 8000 ≥ 2.50	2000 0.63	NA	SYN	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Gen	2000 1.88	1500 0.47	1.00	ADD	750 ≥ 2.50	2000 0.63	NA	SYN	2000 ≥ 2.50	1000 0.32	NA	ADD
	EO Gen	2000 1.88	1000 0.32	0.67	ADD	630 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
<i>A. betulina</i> + penicillin G	Aq Pen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Pen	2000 ≥ 2.50	2000 0.63	1.25	IND	750 ≥ 2.50	1000 0.32	1.46	IND	2000 ≥ 2.50	1500 0.47	0.94	ADD
	EO Pen	2000 ≥ 2.50	1000 0.32	0.63	ADD	630 ≥ 2.50	750 0.23	1.29	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
<i>A. betulina</i> + tetracycline	Aq Tet	≥ 8000 0.23	500 0.16	0.76	ADD	≥ 8000 0.16	190 0.12	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Tet	2000 0.23	500 0.16	0.95	ADD	750 0.16	190 0.06	0.63	ADD	2000 ≥ 2.50	1000 0.32	NA	ADD
	EO Tet	2000 0.23	500 0.16	0.95	ADD	630 0.16	130 0.04	0.46	SYN	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction.

Table 4.2. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *A. betulina* with the various antibiotics, against the Gram-negative pathogens.

Combination	Sample type	<i>E. coli</i> (ATCC 25922)				<i>K. pneumoniae</i> (ATCC 13883)				<i>P. aeruginosa</i> (ATCC 27853)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. betulina</i> + ciprofloxacin	Aq Cip	≥ 8000 0.08	90 0.03	NA	SYN	≥ 8000 0.63	1500 0.47	NA	ADD	≥ 8000 0.16	500 0.16	NA	IND
	Org Cip	≥ 8000 0.08	50 0.02	NA	SYN	≥ 8000 0.63	750 0.23	NA	SYN	4000 0.16	250 0.08	0.56	ADD
	EO Cip	≥ 8000 0.08	70 0.02	NA	SYN	≥ 8000 0.63	190 0.06	NA	SYN	4000 0.16	500 0.16	1.13	IND
<i>A. betulina</i> + gentamicin	Aq Gen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	1000 0.32	NA	SYN	≥ 8000 0.32	750 0.23	NA	ADD
	Org Gen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	2000 0.63	NA	SYN	4000 0.32	500 0.16	0.65	ADD
	EO Gen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 2.50	NA	ADD	4000 0.32	500 0.16	0.65	ADD
<i>A. betulina</i> + tetracycline	Aq Tet	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Tet	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	3000 0.94	NA	IND	4000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND
	EO Tet	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	4000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction.

Agathosma betulina is usually either ingested orally as an aqueous infusion or an alcoholic tincture, for the treatment of UTI's (Appendix F.1). Therefore, the aqueous and organic extract would most closely depict the results seen with the traditional forms of consumption of the plant.

Interestingly, when tested individually, the plant extracts showed MIC values of ≥ 8.00 mg/ml (Table 4.2), thereby demonstrating very weak antimicrobial activity against *E. coli*, which does not support the traditional claims of the effectiveness of *A. betulina* in UTI treatment. This finding is, however, in accordance with other studies, where the aqueous and organic extract, as well as the essential oil of *A. betulina*, demonstrated weak antimicrobial activity against common UTI-causing pathogens, including *E. coli* (Lis-Balchin *et al.*, 2001; Scott and Springfield, 2004a).

When in combination with ciprofloxacin, the extracts both exhibited noteworthy antimicrobial activity, thereby demonstrating synergy. The essential oil also demonstrated a synergistic effect, but is not used traditionally for the treatment of UTI's (Table 4.2).

The aqueous extract of *A. betulina* showed a promising synergistic effect in combination with ciprofloxacin, when tested against *E. coli*. The MIC values of the aqueous extract (90 μ g/ml) and ciprofloxacin (0.03 μ g/ml) in combination were well below the MIC values for the agents when tested individually (≥ 8.00 mg/ml for the aqueous extract and 0.08 μ g/ml for ciprofloxacin), thereby demonstrating a tentative Σ FIC interpretation of synergy (Table 4.2).

When the organic extract of *A. betulina* was combined with ciprofloxacin and tested against *E. coli*, a tentative Σ FIC interpretation of synergy was identified (Table 4.2). The MIC values for the individual agents were ≥ 8.00 mg/ml and 0.08 μ g/ml for the organic extract and antibiotic, respectively. In combination, the MIC values were reduced to 50 μ g/ml and 0.02 μ g/ml for the organic extract and antibiotic, respectively. Therefore, the MIC values for the agents in combination were well below the MIC values of the agents when tested individually, thereby demonstrating a synergistic effect (Table 4.2).

The essential oil of *A. betulina* in combination with ciprofloxacin, when tested against *E. coli*, demonstrated a tentative synergistic interaction, since the MIC values of the agents in combination (70 μ g/ml for the essential oil and 0.02 μ g/ml for ciprofloxacin) were well below the MIC values for the agents when tested individually (≥ 8.00 mg/ml for the essential oil and 0.08 μ g/ml for ciprofloxacin) (Table 4.2). However, this interaction would not be

relevant for the treatment of UTI's, since the essential oil is not used traditionally for this infection (Appendix F.1).

Since the combination between *A. betulina* (essential oil, aqueous and organic extract) and ciprofloxacin against *E. coli* provided such a notable synergistic profile, the combinations were tested at varying ratios to determine the effects of varied concentrations of the combination. The graphical representation (isobolograms) of the interactive profiles for each ratio provides an understanding as to whether the synergistic effect is dose-dependent. The construction of an isobologram also allows for the determining of the agent within a combination that is most responsible for the favourable interaction.

Most ratios were found in the synergistic or additive region of the isobologram (Figure 4.1), with only four combination ratios (ciprofloxacin: *A. betulina* 90:10; 80:20; 70:30 and 30:70 μ l) of the organic extract and one ratio (70:30 μ l) of the essential oil, found in the region indicating an indifferent interaction (refer to Table 2.4 for ratio concentrations).

Six ratios (ciprofloxacin: *A. betulina* 90:10; 80:20; 60:40; 50:50; 30:70 and 20:80 μ l) for the aqueous extract combination and four ratios (90:10; 80:20; 50:50 and 40:60 μ l) for the essential oil combination were found in the synergistic region (refer to Table 2.4 for ratio concentrations); however, no ratios for the organic extract combination were found to be synergistic (Figure 4.1). The varied ratio studies were therefore mostly in accordance with the Σ FIC evaluations which indicated a synergistic interaction for these combinations. However, this is not true for the organic extract combination with ciprofloxacin when tested in varied ratios, where the synergistic profile in Σ FIC evaluation was not depicted in the isobologram (Table 4.2; Figure 4.1).

Resistance of *E. coli* toward ciprofloxacin is an increasing concern throughout the world, particularly with reference to UTI's. The resistance of *E. coli* is also spreading to other antimicrobial treatments for UTI's and therefore there is an urgent need to identify an alternative treatment for these infections (Karlowsky *et al.*, 2002; Arslan *et al.*, 2005).

The identified synergistic interactions between the traditional and conventional form of UTI treatment could ultimately lead to more effective results for UTI infections, by enhancing the efficacy of the antibiotic and possibly assist in preventing the resistance of *E. coli* towards ciprofloxacin. This possible alternative treatment for UTI's caused by resistant *E. coli* requires further *in vivo* testing, to support the *in vitro* results observed here.

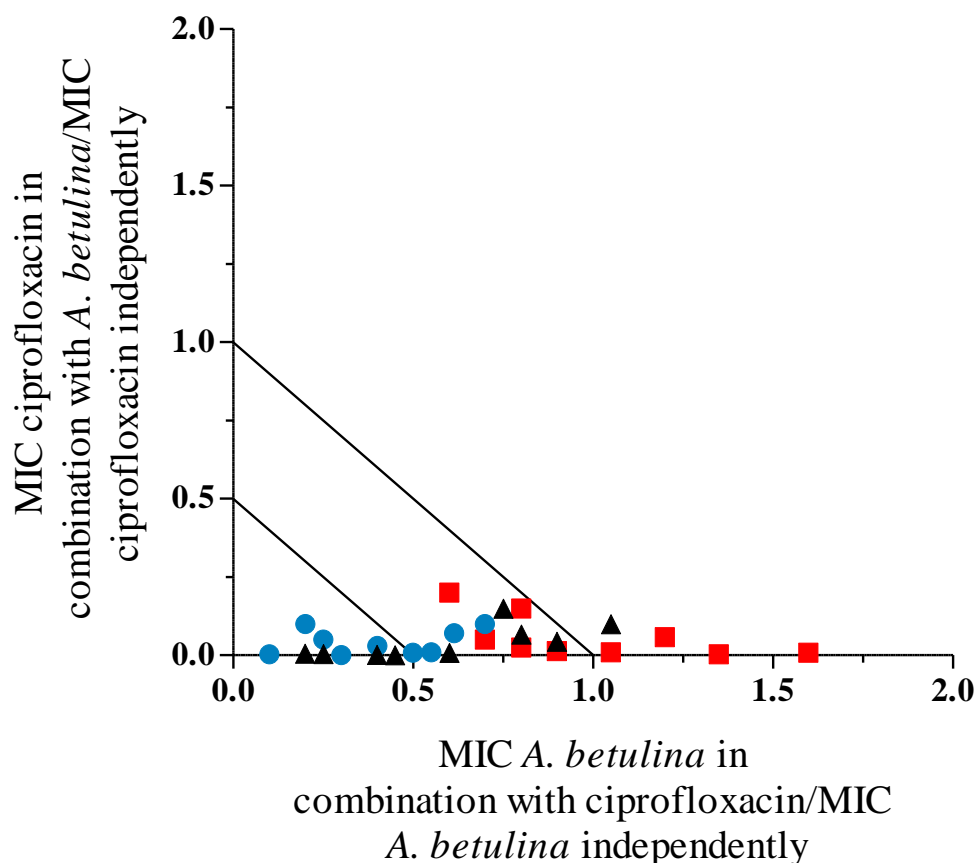


Figure 4.1. Isobologram for *A. betulina* (●= aqueous extract; ■= organic extract; ▲= essential oil) in combination with ciprofloxacin, when tested at various ratios, against *E. coli*.

When the combinations were tested against the two yeasts (Table 4.3), no synergistic interactions were identified and three antagonistic interactions (*A. betulina* essential oil, aqueous and organic extract combined with amphotericin B) were noted against *C. albicans*. Therefore, the possibility for an antagonistic interaction is higher when combining antifungal agents with *A. betulina*. The noted antagonism could reduce the efficacy of the conventional antifungal agent and therefore the concurrent use of these combinations should be cautioned until further *in vivo* testing. The majority of the combinations were, however, found to be indifferent or additive in nature, when tested against the two yeasts (Table 4.3).

Table 4.3. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *A. betulina* with the antifungal agents, against the yeasts.

Combination	Sample type	<i>C. albicans</i> (ATCC 10231)				<i>C. neoformans</i> (ATCC 14116)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. betulina</i> + amphotericin	Aq Amp	6000 1.56	≥ 4000 ≥ 12.50	NA	ANT	3000 0.39	130 0.39	1.04	IND
	Org Amp	3000 1.56	1500 4.69	3.51	ANT	750 0.39	250 0.78	2.33	IND
	EO Amp	2000 1.56	2000 6.25	5.01	ANT	750 0.39	139 0.39	1.17	IND
<i>A. betulina</i> + nystatin	Aq Nys	6000 2.34	380 1.17	0.56	ADD	3000 1.56	380 1.17	0.88	ADD
	Org Nys	3000 2.34	1000 3.13	1.67	IND	750 1.56	500 1.56	1.67	IND
	EO Nys	2000 2.34	1000 3.13	1.84	IND	750 1.56	380 1.17	1.26	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; ANT = antagonistic interaction.

4.2.2. Combinations containing *Aloe ferox*

The combination of *A. ferox* (aqueous and organic extract) with the conventional antibiotics provided a mostly additive interactive profile against the tested Gram-positive pathogens (Table 4.4). Two synergistic interactions were identified, both against *B. cereus*, when the organic extract of *A. ferox* was combined with erythromycin and penicillin G (ΣFIC of 0.34 and 0.35, respectively). Two tentative antagonistic interactions were identified, namely for *A. ferox* aqueous extract in combination with tetracycline, when tested against *S. aureus* and *B. cereus*. All the *A. ferox*: antibiotic combinations tested against *E. faecalis* provided additive interactions (Table 4.4).

Against the tested Gram-negative micro-organisms (Table 4.5), the combination of *A. ferox* (aqueous and organic extract) with the conventional antibiotics exhibited a mostly additive and indifferent profile, with only one synergistic interaction identified (*A. ferox* organic extract with ciprofloxacin against *E. coli*), which was a tentative interpretation. Three antagonistic interactions were found (*A. ferox* aqueous extract and ciprofloxacin against *E. coli*; *A. ferox* aqueous and organic extract together with gentamicin against *P. aeruginosa*) (Table 4.5).

Table 4.4. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *A. ferox* with the various antibiotics, against the Gram-positive pathogens.

Combination	Sample type	<i>S. aureus</i> (ATCC 25923)				<i>B. cereus</i> (ATCC 11778)				<i>E. faecalis</i> (ATCC 29212)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. ferox</i> + ciprofloxacin	Aq Cip	≥ 8000 0.47	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 0.63	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	2000 0.63	NA	ADD
	Org Cip	4000 0.47	1000 0.32	0.93	ADD	3000 0.63	2000 0.63	1.67	IND	≥ 8000 1.25	2000 0.63	NA	ADD
<i>A. ferox</i> + erythromycin	Aq Ery	≥ 8000 0.32	1000 0.32	NA	IND	≥ 8000 0.32	500 0.16	NA	ADD	≥ 8000 1.25	2000 0.63	NA	ADD
	Org Ery	4000 0.32	1000 0.32	1.28	IND	3000 0.32	500 0.16	0.34	SYN	≥ 8000 1.25	2000 0.63	NA	ADD
<i>A. ferox</i> + gentamicin	Aq Gen	≥ 8000 1.88	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	3000 0.94	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Gen	4000 1.88	1500 0.47	1.00	ADD	3000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
<i>A. ferox</i> + penicillin G	Aq Pen	≥ 8000 ≥ 2.50	2000 0.63	NA	ADD	≥ 8000 ≥ 2.50	2000 0.63	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Pen	4000 ≥ 2.50	2000 0.63	0.75	ADD	3000 ≥ 2.50	750 0.24	0.35	SYN	≥ 8000 ≥ 2.50	15000 0.47	0.94	ADD
<i>A. ferox</i> + tetracycline	Aq Tet	≥ 8000 0.23	≥ 4000 ≥ 1.25	NA	<i>ANT</i>	≥ 8000 0.16	≥ 4000 ≥ 1.25	NA	<i>ANT</i>	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Tet	4000 0.23	750 0.24	1.23	IND	3000 0.16	190 0.06	0.63	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction.

Table 4.5. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *A. ferox* with the various antibiotics, against the Gram-negative pathogens.

Combination	Sample type	<i>E. coli</i> (ATCC 25922)				<i>K. pneumoniae</i> (ATCC 13883)				<i>P. aeruginosa</i> (ATCC 27853)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. ferox</i> + ciprofloxacin	Aq Cip	≥ 8000 0.08	2000 0.63	NA	ANT	≥ 8000 0.63	≥ 4000 ≥ 1.25	NA	IND	6000 0.16	1500 0.47	3.19	IND
	Org Cip	≥ 8000 0.08	50 0.02	NA	SYN	≥ 8000 0.63	3000 0.94	NA	IND	6000 0.16	750 0.23	1.57	IND
<i>A. ferox</i> + gentamicin	Aq Gen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	3000 0.94	NA	ADD	6000 0.32	≥ 4000 ≥ 1.25	NA	ANT
	Org Gen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	6000 0.32	≥ 4000 ≥ 1.25	NA	ANT
<i>A. ferox</i> + tetracycline	Aq Tet	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	6000 ≥ 2.50	2000 0.63	NA	ADD
	Org Tet	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	2000 0.63	NA	ADD	6000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; **ANT** = antagonistic interaction.

Interestingly, the aqueous extract when tested in combination with ciprofloxacin against *E. coli*, exhibited a tentative synergistic interaction; however, the organic extract combination against *E. coli* showed a tentative antagonistic interaction (Table 4.5).

When the combinations of *A. ferox* (aqueous and organic extract) with the conventional antifungals were tested against the yeasts (Table 4.6), no synergy was noted for any of the combinations. However, four combinations were found to be antagonistic in nature (*A. ferox* aqueous extract with amphotericin B against *C. albicans* and *C. neoformans*; *A. ferox* aqueous extract with nystatin against *C. albicans* and *C. neoformans*). The antagonism was only noted for the aqueous extract combinations, with the organic extract combinations demonstrating either an additive or indifferent profile (Table 4.6).

Table 4.6. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *A. ferox* with the various antifungal agents, against the yeasts.

Combination	Sample type	<i>C. albicans</i> (ATCC 10231)				<i>C. neoformans</i> (ATCC 14116)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. ferox</i> + amphotericin	Aq Amp	≥ 8000 1.56	3000 9.38	NA	ANT	≥ 8000 0.39	≥ 4000 ≥ 1.25	NA	ANT
	Org Amp	2000 1.56	1500 4.69	3.51	IND	≥ 8000 0.39	130 0.39	1.02	IND
<i>A. ferox</i> + nystatin	Aq Nys	≥ 8000 2.34	≥ 4000 ≥ 12.50	NA	ANT	≥ 8000 1.56	≥ 4000 ≥ 12.50	NA	ANT
	Org Nys	2000 2.34	1000 3.13	1.84	IND	≥ 8000 1.56	250 0.78	0.53	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; SYN = synergistic interaction; ANT = antagonistic interaction.

4.2.3. Combinations containing *Artemisia afra*

The combination of *A. afra* (essential oil, aqueous and organic extract) with the conventional antibiotics demonstrated a mostly additive or indifferent interactive profile, when tested against the Gram-positive micro-organisms (Table 4.7). Three antagonistic interactions were identified, which was seen with the aqueous extract of *A. afra* when combined with ciprofloxacin and tested against *S. aureus* (tentative antagonistic interaction) and when the organic extract of *A. afra* was combined with penicillin G and tested against *S. aureus* (ΣFIC of 4.25) and *B. cereus* (ΣFIC of 4.40) (Table 4.7).

Table 4.7. MIC (µg/ml) and ΣFIC values for the combination of *A. afra* with the various antibiotics, against the Gram-positive pathogens.

Combination	Sample type	<i>S. aureus</i> (ATCC 25923)				<i>B. cereus</i> (ATCC 11778)				<i>E. faecalis</i> (ATCC 29212)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. afra</i> + ciprofloxacin	Aq Cip	2000 0.47	≥ 4000 ≥ 1.25	NA	ANT	≥ 8000 0.63	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND
	Org Cip	500 0.47	500 0.16	1.34	IND	380 0.63	500 0.16	1.57	IND	2000 1.25	2000 0.63	1.50	IND
	EO Cip	2000 0.47	2000 0.63	2.34	IND	2000 0.63	1000 0.32	1.01	IND	≥ 8000 1.25	2000 0.63	NA	ADD
<i>A. afra</i> + erythromycin	Aq Ery	2000 0.32	1000 0.32	1.53	IND	≥ 8000 0.32	750 0.23	NA	ADD	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND
	Org Ery	500 0.32	500 0.16	1.52	IND	380 0.32	250 0.08	0.92	ADD	2000 1.25	2000 0.63	1.50	IND
	EO Ery	2000 0.32	1000 0.32	1.53	IND	2000 0.32	500 0.16	0.77	ADD	≥ 8000 1.25	2000 0.63	NA	ADD
<i>A. afra</i> + gentamicin	Aq Gen	2000 1.88	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Gen	500 1.88	1000 0.32	2.17	IND	380 ≥ 2.50	1000 0.32	NA	SYN	2000 ≥ 2.50	1000 0.32	NA	ADD
	EO Gen	2000 1.88	1000 0.32	0.67	ADD	2000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
<i>A. afra</i> + penicillin G	Aq Pen	2000 ≥ 2.50	1500 0.47	0.94	ADD	≥ 8000 ≥ 2.50	750 0.24	NA	SYN	≥ 8000 ≥ 2.50	1000 0.32	NA	SYN
	Org Pen	500 ≥ 2.50	2000 0.63	4.25	ANT	380 ≥ 2.50	1500 0.47	4.40	ANT	2000 ≥ 2.50	2000 0.63	1.25	IND
	EO Pen	2000 ≥ 2.50	2000 0.63	1.25	IND	2000 ≥ 2.50	1000 0.32	0.63	ADD	≥ 8000 ≥ 2.50	2000 0.63	NA	ADD
<i>A. afra</i> + tetracycline	Aq Tet	2000 0.23	1000 0.32	1.89	IND	≥ 8000 0.16	190 0.06	NA	SYN	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Tet	500 0.23	500 0.16	1.70	IND	380 0.16	130 0.04	0.59	ADD	2000 ≥ 2.50	1000 0.32	NA	ADD
	EO Tet	2000 0.23	500 0.16	0.95	ADD	2000 0.16	190 0.06	0.48	SYN	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where ≥ MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction.

Five synergistic combinations were seen when *A. afra* (essential oil, aqueous and organic extract) was combined with the conventional antibiotics and tested against the selected Gram-positive pathogens. Four of these interactions (*A. afra* aqueous extract with penicillin G against *B. cereus* and *E. faecalis*; *A. afra* organic extract and gentamicin against *B. cereus* and the combination of *A. afra* aqueous extract and tetracycline against *B. cereus*) were tentative interpretations. The final synergistic interaction was found with the combination of the essential oil of *A. afra* and tetracycline, against *B. cereus*, with a Σ FIC of 0.48 (Table 4.7).

When the combinations with *A. afra* were tested against the Gram-negative pathogens (Table 4.8), a mostly additive and indifferent interactive profile was noted. Three synergistic interactions were identified, namely *A. afra* organic extract and essential oil in combination with ciprofloxacin against *E. coli* (Σ FIC of 0.27 for both combinations) and the combination of *A. afra* organic extract with tetracycline against *P. aeruginosa*, with a tentative interpretation of synergy. One antagonistic interaction was noted between the aqueous extract of *A. afra* and ciprofloxacin, against *E. coli* (Σ FIC of 8.55), which is interesting, since the organic extract and essential oil combinations with ciprofloxacin demonstrated a synergistic interaction against *E. coli*. This variation was studied further by examining varied ratios of the combination. *Escherichia coli* commonly causes infectious GI complaints, which could arise from eating or drinking contaminated food or water (Merck Manual, 2006). In rural areas, these GI complaints are commonly treated with *A. afra* (Appendix F.3) in comparison to ciprofloxacin usage in orthodox medicine (Merck Manual, 2006). There is, therefore, a high probability for concurrent use of these two forms of healthcare. *Artemisia afra* is commonly consumed orally as an aqueous infusion (herbal tea) for GI complaints. The MIC value for the aqueous extract (2.00 mg/ml) in combination with ciprofloxacin was below the MIC value when tested individually (3.00 mg/ml); however, the MIC value for ciprofloxacin (0.63 μ g/ml) in combination was far greater than when tested individually (0.08 μ g/ml), resulting in an antagonistic profile for the combination (Table 4.8). Therefore, it is likely that the aqueous extract of *A. afra* interacted with ciprofloxacin, making it less active against *E. coli* (Table 4.8). Therefore concurrent use should be cautioned and further pharmacokinetic studies undertaken to investigate the mechanism of the interaction. These studies would most likely involve animal studies, where absorption is analysed through plasma/blood curve-time data; metabolism analysed through metabolite plasma profile studies; and excretion analysed through urine or faecal sample data (Japanese Ministry of Health, Labour and Welfare, 2001).

Table 4.8. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *A. afra* with the various antibiotics, against the Gram-negative pathogens.

Combination	Sample type	<i>E. coli</i> (ATCC 25922)				<i>K. pneumoniae</i> (ATCC 13883)				<i>P. aeruginosa</i> (ATCC 27853)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. afra</i> + ciprofloxacin	Aq Cip	3000 0.08	2000 0.63	8.55	ANT	4000 0.63	3000 0.94	2.24	IND	2000 0.16	1500 0.47	3.69	IND
	Org Cip	3000 0.08	70 0.02	0.27	SYN	2000 0.63	750 0.23	0.75	ADD	1500 0.16	250 0.08	0.67	ADD
	EO Cip	3000 0.08	70 0.02	0.27	SYN	≥ 8000 0.63	1000 0.32	NA	ADD	4000 0.16	500 0.16	1.13	IND
<i>A. afra</i> + gentamicin	Aq Gen	3000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	4000 ≥ 2.50	2000 0.63	NA	ADD	2000 0.32	1500 0.47	2.27	IND
	Org Gen	3000 ≥ 2.50	2000 0.63	0.92	ADD	2000 ≥ 2.50	1000 0.32	NA	ADD	1500 0.32	500 0.16	0.85	ADD
	EO Gen	3000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	4000 0.32	500 0.16	0.65	ADD
<i>A. afra</i> + tetracycline	Aq Tet	3000 1.25	≥ 4000 ≥ 1.25	NA	IND	4000 1.25	≥ 4000 ≥ 1.25	NA	IND	2000 ≥ 2.50	2000 0.63	NA	IND
	Org Tet	3000 1.25	≥ 4000 ≥ 1.25	NA	IND	2000 1.25	3000 0.94	2.25	IND	1500 ≥ 2.50	1000 0.32	NA	SYN
	EO Tet	3000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	4000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction.

The combination of essential oil, aqueous or organic extract with ciprofloxacin, was tested in various ratios against *E. coli*, since these combinations showed variance in interactive profiles, ranging from synergistic to highly antagonistic. In the varied ratio studies (Figure 4.2), the Σ FIC evaluation of antagonism (Table 4.8) for the aqueous extract combination was supported by the ratio containing the equal volumes (50:50 μ l).

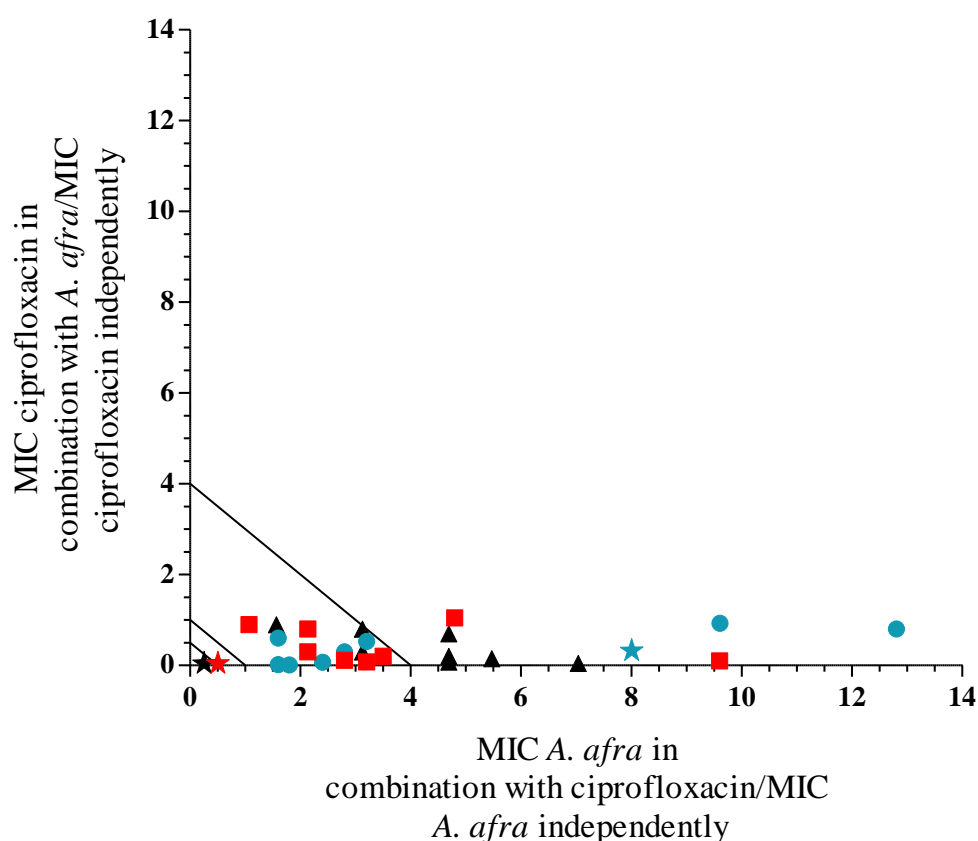


Figure 4.2. Isobologram for *A. afra* (● = aqueous extract; ■ = organic extract; ▲ = essential oil) in combination with ciprofloxacin, when tested at various ratios, against *E. coli* (★ = 50:50 μ l ratio for aqueous extract; ★ = 50:50 μ l ratio for organic extract; ★ = 50:50 μ l ratio for essential oil).

The Σ FIC evaluation of synergy (Table 4.8) for the organic extract combination was again supported in the varied ratio study (Figure 4.2) by the ratio containing equal volumes of each agent in the combination (50:50 μ l). However, the synergistic profile for the essential oil as seen in the Σ FIC evaluation (Table 4.8) was not supported in the varied ratio study, where all ratio points were either found in the indifferent or antagonistic region (Figure 4.2). Interestingly, the ratio 30:70 μ l (ciprofloxacin: *A. afra*) was found in the antagonistic region,

for all three plant sample types (essential oil, aqueous and organic extract) prepared from *A. afra* (refer to Table 2.4 for ratio concentrations).

The combination of the essential oil, aqueous and organic extract of *A. afra* together with the conventional antifungal agents, when tested against the yeasts (Table 4.9), demonstrated no synergistic interactions. Three antagonistic interactions were identified, all of which contained amphotericin B and when tested against *C. albicans* (Σ FIC of 4.51, 6.76 and 5.34 for the essential oil, aqueous and organic extract combination, respectively) (Table 4.9).

Table 4.9. MIC (μ g/ml) and Σ FIC values for the combination of *A. afra* with the various antifungal agents, against the yeasts.

Combination	Sample type	<i>C. albicans</i> (ATCC 10231)				<i>C. neoformans</i> (ATCC 14116)			
		Ind. MIC	Combo. MIC	Σ FIC	Int.	Ind. MIC	Combo. MIC	Σ FIC	Int.
<i>A. afra</i> + amphotericin	Aq Amp	4000 1.56	3000 9.38	6.76	ANT	1000 0.39	190 0.59	1.69	IND
	Org Amp	1500 1.56	2000 6.25	5.34	ANT	750 0.39	190 0.59	1.75	IND
	EO Amp	1000 1.56	1500 4.69	4.51	ANT	750 0.39	250 0.78	2.33	IND
<i>A. afra</i> + nystatin	Aq Nys	4000 2.34	750 2.35	1.19	IND	1000 1.56	190 0.59	0.57	ADD
	Org Nys	1500 2.34	380 1.17	0.75	ADD	750 1.56	500 1.56	1.67	IND
	EO Nys	1000 2.34	1000 3.13	2.34	IND	750 1.56	500 1.56	1.67	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; ADD = additive interaction; IND = indifferent interaction; ANT = antagonistic interaction.

4.2.4. Combinations containing *Aspalathus linearis*

The combinations of the aqueous and organic extracts of *A. linearis* with the conventional antibiotics, when tested against the Gram-positive pathogens (Table 4.10), demonstrated no antagonistic interactions. There were, however, ten identified synergistic interactions. The organic extract of *A. linearis* in combination with gentamicin demonstrated synergy against all three of the tested Gram-positive pathogens (Σ FIC of 0.50 against *S. aureus* and a tentative interpretation of synergy against *B. cereus* and *E. faecalis*). The aqueous extract of *A. linearis* in combination with penicillin G also demonstrated synergy against all three tested Gram-positive pathogens (tentative interpretations of synergy against *S. aureus* and *B. cereus* and a Σ FIC value of 0.46 against *E. faecalis*). The organic extract of *A. linearis* in

Table 4.10. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *A. linearis* with the various antibiotics, against the Gram-positive pathogens.

Combination	Sample type	<i>S. aureus</i> (ATCC 25923)				<i>B. cereus</i> (ATCC 11778)				<i>E. faecalis</i> (ATCC 29212)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. linearis</i> + ciprofloxacin	Aq Cip	≥ 8000 0.47	2000 0.63	NA	IND	≥ 8000 0.63	2000 0.63	NA	IND	3000 1.25	2000 0.63	1.17	IND
	Org Cip	3000 0.47	500 0.16	0.51	ADD	2000 0.63	1000 0.32	1.01	IND	2000 1.25	1500 0.47	1.13	IND
<i>A. linearis</i> + erythromycin	Aq Ery	≥ 8000 0.32	2000 0.63	NA	IND	≥ 8000 0.32	1000 0.32	NA	IND	3000 1.25	4000 1.25	NA	IND
	Org Ery	3000 0.32	2000 0.63	2.70	IND	2000 0.32	500 0.16	0.77	ADD	2000 1.25	≥ 4000 ≥ 1.25	NA	IND
<i>A. linearis</i> + gentamicin	Aq Gen	≥ 8000 1.88	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	3000 0.94	NA	IND	3000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND
	Org Gen	3000 1.88	1000 0.32	0.50	SYN	2000 ≥ 2.50	500 0.16	NA	SYN	2000 ≥ 2.50	500 0.16	NA	SYN
<i>A. linearis</i> + penicillin G	Aq Pen	≥ 8000 ≥ 2.50	30 0.01	NA	SYN	≥ 8000 ≥ 2.50	250 0.08	NA	SYN	3000 ≥ 2.50	1000 0.32	0.46	SYN
	Org Pen	3000 ≥ 2.50	30 0.01	0.01	SYN	2000 ≥ 2.50	130 0.04	0.08	SYN	2000 ≥ 2.50	1500 0.47	0.94	ADD
<i>A. linearis</i> + tetracycline	Aq Tet	≥ 8000 0.23	1000 0.32	NA	IND	≥ 8000 0.16	250 0.08	NA	ADD	3000 ≥ 2.50	3000 0.94	NA	IND
	Org Tet	3000 0.23	500 0.16	0.87	ADD	2000 0.16	190 0.06	0.48	SYN	2000 ≥ 2.50	2000 0.63	NA	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction.

combination with penicillin G demonstrated synergy, however, only against *S. aureus* (Σ FIC of 0.01) and *B. cereus* (Σ FIC of 0.08). Tiwari *et al.* (2005) also investigated the combination of other green and black teas with antimicrobials, chloramphenicol, gentamicin, methicillin and nalidixic acid, where a mostly synergistic profile was also seen against enteropathogens. Against *E. faecalis*, the combination demonstrated an additive effect (Σ FIC of 0.94). Another synergistic interaction was identified between the organic extract of *A. linearis* and tetracycline, against *B. cereus* (Σ FIC of 0.48) (Table 4.10).

Aspalathus linearis is the plant from which the popular beverage, rooibos tea, is derived. This beverage is consumed by many people throughout the world, not only for the aroma and pleasant taste, but for its medicinal properties too (Joubert *et al.*, 2008; Van Wyk *et al.*, 2009). Therefore, the concurrent consumption with conventional antimicrobials has been considered in this study. The common form of consumption of *A. linearis* is via the preparation of a herbal tea, where water is used for the extraction process. Therefore, the interactions with the aqueous extract of *A. linearis* would be most relevant. Since the combination of the aqueous and organic extract of *A. linearis* with penicillin G demonstrated the most synergistic profile against the Gram-positive pathogens, it is highly likely that one of the constituents of the extracts could have potentiated the uptake and antimicrobial effect of the antibiotic. Since this combination was so notable, a varied ratio study was undertaken for these two combinations. Rooibos is not consumed in fixed concentrations, since people vary the number of cups of tea that they drink. Also, the concentration can vary depending on the time that the tea bag is left to draw. The active ingredients of the tea leaves can also be affected by variations in the season, cultivation, harvesting and leaf preparation. Therefore, the varied ratio studies would provide one with an indication of the effect of varied concentrations on the interactive profile and whether the synergistic effect is dose-dependent. During the varied ratio studies (Figure 4.3), the synergistic interactions found in the Σ FIC evaluation (Table 4.10) were mostly supported. For the combination of *A. linearis* aqueous extract and penicillin G (Figure 4.3.a), all ratios were found to be either synergistic or additive in nature, with no ratios falling above the 1.0:1.0 line. The penicillin G: *A. linearis* aqueous extract ratio, consisting of equal volumes of each agent in the combination (5:5), corresponding to a concentration of 0.005 mg/ml:16.00 mg/ml (Table 2.4) was found to be synergistic against all three Gram-positive pathogens. The combination was most synergistic against *S. aureus*, with six of the nine ratios demonstrating synergy (Figure 4.3.a). The three

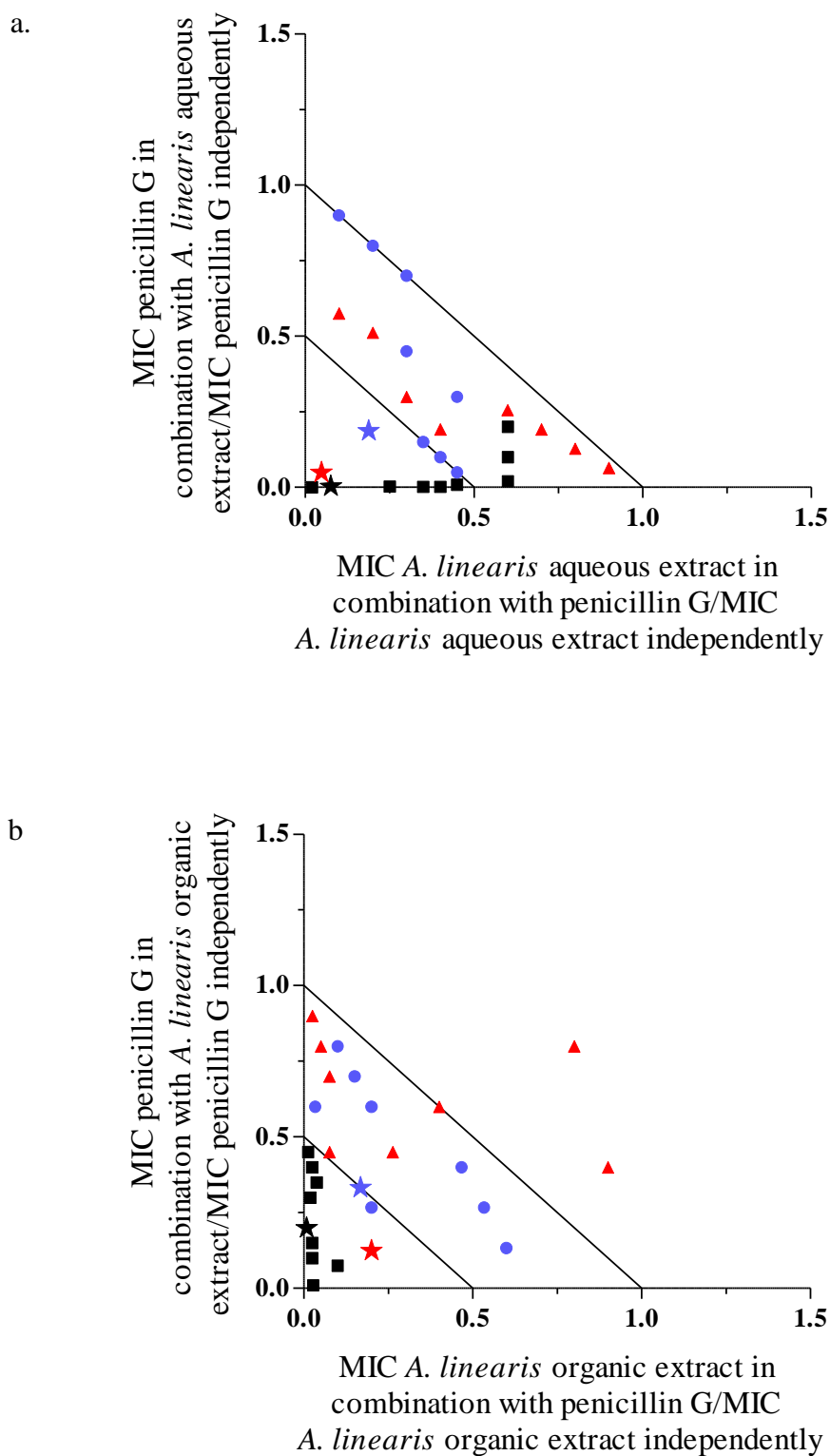


Figure 4.3. Isobologram for *A. linearis* aqueous (a) and organic (b) extract in combination with penicillin G, when tested at various ratios, against the Gram-positive micro-organisms (■ = *S. aureus*; ▲ = *B. cereus*; ● = *E. faecalis*; ★ = 50:50 μ l ratio against *S. aureus*; ★ = 50:50 μ l ratio against *B. cereus*; ★ = 50:50 μ l ratio against *E. faecalis*).

penicillin G: *A. linearis* ratios for the aqueous extract combination that were not found to be synergistic against *S. aureus*, were the 30:70; 20:80 and 10:90 μ l ratios, which were highest in *A. linearis* aqueous extract volume, as opposed to penicillin G, with concentrations of 0.003 mg/ml:22.40 mg/ml; 0.002 mg/ml:25.60 mg/ml and 0.001 mg/ml:28.80 mg/ml, for the three ratios, respectively (Table 2.4).

For the varied ratio combinations of the organic extract of *A. linearis* and penicillin G, all ratios were found to be either synergistic or additive, except for two penicillin G: *A. linearis* ratios, namely the 90:10 and 80:20 μ l ratios against *B. cereus*, which corresponds to concentrations of 0.009 mg/ml:3.20 mg/ml and 0.008 mg/ml:6.40 mg/ml, respectively, and were identified as being indifferent in nature (Table 4.10). Similar to the aqueous extract combination, the penicillin G: *A. linearis* organic extract ratio, consisting of equal volumes of each agent in the combination (50:50 μ l), which corresponds to a concentration of 0.005 mg/ml:16.00 mg/ml (Table 2.4), was found to be synergistic against all three Gram-positive pathogens. Similarly to the aqueous extract, the organic extract combination with penicillin G in varied ratios, was most synergistic against *S. aureus*, where all nine of the tested ratios were found below the 0.5:0.5 line (Figure 4.3.b). One cup of rooibos tea has been estimated to have an average concentration of 2 mg/ml (Schepers, 2001), therefore, the ratio containing equal volumes of extract and penicillin would require about eight cups of tea to be consumed with 0.005 mg/ml of penicillin G (Table 2.4) for the strong synergistic interaction to occur.

Studies have found that epigallocatechin-3-gallate (EGEG), a compound found in many teas, has a synergistic effect with β -lactam antibiotics, such as penicillin and its derivatives, when tested against MRSA. EGEG is not present in rooibos tea (Almajano *et al.*, 2008), but it does support further studies to identify the compound and mechanism of action by which this strong synergistic interaction is facilitated. Many studies reviewed by Van Vuuren and Viljoen (2011) reported that penicillin G possessed a potentiating or synergistic effect with other plants, such as *Catha edulis*, where a 4-fold potentiation of penicillin G against *Fusobacterium nucleatum* was seen (Al-hebshi *et al.*, 2006). *Rhus coriaria*, *Sacropoterium spinosum* and *Rosa damasceana* were also found to be synergistic in combination with penicillin G, when tested against three clinical strains of *P. aeruginosa* (Adwan *et al.*, 2010). Penicillin G has also shown strong potentiating activity when in combination with some plant compounds, such as eugenol, thymol and carvacrol, when tested against *E. coli*, *S. aureus*, *Streptococcus pyogenes* and *Salmonella typhimurium*. The strongest synergistic effect was seen against *S. aureus* (Σ FIC of 0.11) when carvacrol and penicillin G were combined

Table 4.11. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *A. linearis* with the various antibiotics, against the Gram-negative pathogens.

Combination	Sample type	<i>E. coli</i> (ATCC 25922)				<i>K. pneumoniae</i> (ATCC 13883)				<i>P. aeruginosa</i> (ATCC 27853)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>A. linearis</i> + ciprofloxacin	Aq Cip	1500 0.08	1000 0.32	4.67	ANT	4000 0.63	500 0.16	0.64	ADD	≥ 8000 0.16	1500 0.47	3.13	IND
	Org Cip	1500 0.08	500 0.16	2.33	IND	3000 0.63	190 0.06	0.16	SYN	3000 0.16	500 0.16	1.17	IND
<i>A. linearis</i> + gentamicin	Aq Gen	1500 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	4000 ≥ 2.50	2000 0.63	NA	ADD	≥ 8000 0.32	1500 0.47	1.71	IND
	Org Gen	1500 ≥ 2.50	3000 0.94	2.38	IND	3000 ≥ 2.50	2000 0.63	NA	ADD	3000 0.32	≥ 4000 ≥ 1.25	NA	ANT
<i>A. linearis</i> + tetracycline	Aq Tet	1500 1.25	≥ 4000 ≥ 1.25	NA	IND	4000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	1500 0.47	NA	SYN
	Org Tet	1500 1.25	3000 0.94	2.75	IND	3000 1.25	≥ 4000 ≥ 1.25	NA	IND	3000 ≥ 2.50	1500 0.47	NA	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction.

(Palaniappan and Holley, 2010). The combination of *A. linearis* (aqueous and organic extract) with the antibiotics against the Gram-negative pathogens (Table 4.11), demonstrated two antagonistic interactions (*A. linearis* aqueous extract with ciprofloxacin against *E. coli* with a Σ FIC of 4.67 and *A. linearis* organic extract with gentamicin against *P. aeruginosa* with a tentative antagonistic interpretation) (Table 4.11).

Two synergistic interactions were identified (*A. linearis* organic extract with ciprofloxacin against *K. pneumoniae* with a Σ FIC of 0.16 and *A. linearis* aqueous extract with tetracycline against *P. aeruginosa* with a tentative synergistic interpretation) (Table 4.11). Therefore, against the Gram-negative pathogens tested there was an equal potential for either synergistic or antagonistic interactions.

The combinations of *A. linearis* (aqueous and organic extract) with the conventional antifungal agents, when tested against the two yeasts (Table 4.12), demonstrated no synergistic interactions and two tentative antagonistic interactions (*A. linearis* aqueous extract and amphotericin B when tested against *C. albicans* and *C. neoformans*) (Table 4.12).

Table 4.12. MIC (μ g/ml) and Σ FIC values for the combination of *A. linearis* with the various antifungal agents, against the yeasts.

Combination	Sample type	<i>C. albicans</i> (ATCC 10231)				<i>C. neoformans</i> (ATCC 14116)			
		Ind. MIC	Combo. MIC	Σ FIC	Int.	Ind. MIC	Combo. MIC	Σ FIC	Int.
<i>A. linearis</i> + amphotericin	Aq Amp	≥ 8000 1.56	≥ 4000 ≥ 12.50	NA	ANT	≥ 8000 0.39	750 2.35	NA	ANT
	Org Amp	3000 1.56	1500 4.69	3.51	IND	1500 0.39	130 0.39	1.09	IND
<i>A. linearis</i> + nystatin	Aq Nys	≥ 8000 2.34	1500 4.69	NA	IND	≥ 8000 1.56	1500 4.70	NA	IND
	Org Nys	3000 2.34	1000 3.13	1.67	IND	1500 1.56	190 0.59	0.51	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute Σ FIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; ANT = antagonistic interaction.

4.2.5. Combinations containing *Lippia javanica*

The combination of *L. javanica* (essential oil, aqueous and organic extract) with the antibiotics against the Gram-positive pathogens (Table 4.13), demonstrated no antagonistic

Table 4.13. MIC (µg/ml) and ΣFIC values for the combination of *L. javanica* with the various antibiotics, against the Gram-positive pathogens.

Combination	Sample type	<i>S. aureus</i> (ATCC 25923)				<i>B. cereus</i> (ATCC 11778)				<i>E. faecalis</i> (ATCC 29212)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>L. javanica</i> + ciprofloxacin	Aq Cip	4000 0.47	2000 0.63	1.84	IND	≥ 8000 0.63	2000 0.63	NA	IND	≥8000 1.25	≥ 4000 ≥ 1.25	NA	IND
	Org Cip	250 0.47	100 0.05	0.51	ADD	≥ 8000 0.63	130 0.04	NA	SYN	1000 1.25	1000 0.32	1.26	IND
	EO Cip	1500 0.47	500 0.16	0.67	ADD	1500 0.63	500 0.16	0.58	ADD	3000 1.25	750 0.24	0.44	SYN
<i>L. javanica</i> + erythromycin	Aq Ery	4000 0.32	1000 0.32	1.28	IND	≥ 8000 0.32	500 0.16	NA	ADD	≥8000 1.25	2000 0.63	NA	ADD
	Org Ery	250 0.32	500 0.16	2.52	IND	≥ 8000 0.32	20 0.005	NA	SYN	1000 1.25	250 0.08	0.32	SYN
	EO Ery	1500 0.32	1000 0.32	1.70	IND	1500 0.32	190 0.06	0.32	SYN	3000 1.25	3000 0.94	1.75	IND
<i>L. javanica</i> + gentamicin	Aq Gen	4000 1.88	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Gen	250 1.88	130 0.04	0.54	ADD	≥ 8000 ≥ 2.50	130 0.04	NA	SYN	1000 ≥ 2.50	130 0.04	NA	SYN
	EO Gen	1500 1.88	750 0.24	0.63	ADD	1500 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	3000 ≥ 2.50	2000 0.63	NA	ADD
<i>L. javanica</i> + penicillin G	Aq Pen	4000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	2000 0.63	NA	ADD	≥8000 ≥ 2.50	2000 0.63	NA	ADD
	Org Pen	250 ≥ 2.50	750 0.23	3.09	IND	≥ 8000 ≥ 2.50	750 0.24	NA	SYN	1000 ≥ 2.50	2000 0.63	2.25	IND
	EO Pen	1500 ≥ 2.50	1000 0.32	0.80	ADD	1500 ≥ 2.50	1000 0.32	0.80	ADD	3000 ≥ 2.50	2000 0.63	0.92	ADD
<i>L. javanica</i> + tetracycline	Aq Tet	4000 0.23	1000 0.32	1.64	IND	≥ 8000 0.16	250 0.08	NA	ADD	≥8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Tet	250 0.23	380 0.12	2.04	IND	≥ 8000 0.16	20 0.005	NA	SYN	1000 ≥ 2.50	500 0.16	NA	ADD
	EO Tet	1500 0.23	500 0.16	1.03	IND	1500 0.16	130 0.04	0.34	SYN	3000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where ≥ MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction.

interactions, but ten synergistic interactions were identified. Seven of the ten interactions, namely *L. javanica* organic extract with ciprofloxacin (tentative synergy), erythromycin (tentative synergy), gentamicin (tentative synergy), penicillin G (tentative synergy) and tetracycline (Σ FIC of 0.34), along with the essential oil of *L. javanica* with erythromycin (Σ FIC of 0.32) and tetracycline (Σ FIC of 0.34), were against *B. cereus*. Whilst the remaining three synergistic interactions were seen against *E. faecalis*, namely *L. javanica* essential oil with ciprofloxacin (Σ FIC 0.44), *L. javanica* organic extract with erythromycin (Σ FIC of 0.32) and the organic extract with gentamicin (tentative synergistic interaction) (Table 4.13).

The combination of *L. javanica* (essential oil, aqueous and organic extract) with the conventional antibiotics, when tested against the Gram-negative pathogens (Table 4.14), resulted in only one tentative antagonistic interaction, between the aqueous extract and gentamicin against *P. aeruginosa* (Table 4.14).

Six synergistic interactions were identified (Table 4.14). Both the organic extract and essential oil, when combined with ciprofloxacin and tested against *E. coli*, demonstrated a synergistic interaction (Σ FIC of 0.32 and 0.14, respectively). The organic extract with ciprofloxacin also showed synergy against *K. pneumoniae* (Σ FIC of 0.19). When the organic extract of *L. javanica* was combined with gentamicin, synergy was seen against *K. pneumoniae* (tentative synergy) and *P. aeruginosa* (Σ FIC of 0.32). The organic extract combination with tetracycline, also showed synergy against *P. aeruginosa* (tentative synergy) (Table 4.14).

The combination of *L. javanica*, commonly used traditionally for intestinal complaints, with ciprofloxacin provided a notable interactive profile, when tested against *E. coli*. *Lippia javanica* is frequently prepared as a herbal tea for oral ingestion using water for the extraction process, for the treatment of GI complaints (Appendix F.5). Therefore, the aqueous extract results would depict most closely the effects seen with traditional use of the plant and was found to display an indifferent interaction in the current study, with an Σ FIC value of 2.25 against *E. coli* (Table 4.14). The MIC value for the aqueous extract in combination (0.50 mg/ml) was lower than the MIC value when tested individually (2.00 mg/ml) (Table 3.3). However, the MIC value for ciprofloxacin in combination (0.16 μ g/ml) was higher than when tested individually (0.08 μ g/ml). Therefore, an indifferent interaction was noted for this combination, which alleviates some of the concern related to the concurrent use of these two forms of healthcare for GI complaints.

Table 4.14. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *L. javanica* with the various antibiotics, against the Gram-negative pathogens.

Combination	Sample type	<i>E. coli</i> (ATCC 25922)				<i>K. pneumoniae</i> (ATCC 13883)				<i>P. aeruginosa</i> (ATCC 27853)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>L. javanica</i> + ciprofloxacin	Aq Cip	2000 0.08	500 0.16	2.25	IND	≥ 8000 0.63	3000 0.94	NA	ADD	2000 0.16	1000 0.32	2.50	IND
	Org Cip	1000 0.08	70 0.02	0.32	SYN	1000 0.63	130 0.04	0.19	SYN	4000 0.16	250 0.08	0.56	ADD
	EO Cip	2000 0.08	30 0.01	0.14	SYN	3000 0.63	1500 0.47	1.25	IND	2000 0.16	750 0.23	1.82	IND
<i>L. javanica</i> + gentamicin	Aq Gen	2000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	2000 0.32	≥ 4000 ≥ 1.25	NA	ANT
	Org Gen	1000 ≥ 2.50	3000 0.94	2.38	IND	1000 ≥ 2.50	250 0.08	NA	SYN	4000 0.32	250 0.08	0.32	SYN
	EO Gen	2000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	3000 ≥ 2.50	2000 0.63	NA	ADD	2000 0.32	500 0.16	0.77	ADD
<i>L. javanica</i> + tetracycline	Aq Tet	2000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	3000 0.94	NA	IND	2000 ≥ 2.50	3000 0.94	NA	IND
	Org Tet	1000 1.25	2000 0.63	2.50	IND	1000 1.25	1500 0.47	1.88	IND	4000 ≥ 2.50	500 0.16	NA	SYN
	EO Tet	2000 1.25	2000 0.63	1.50	IND	3000 1.25	≥ 4000 ≥ 1.25	NA	IND	2000 ≥ 2.50	1500 0.47	NA	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction.

The organic extract and essential oil combinations with ciprofloxacin when tested against *E. coli*, demonstrated a very different interaction to that of the aqueous extract, where a synergistic interactive profile was observed, with Σ FIC values of 0.32 and 0.14, respectively (Table 4.14).

The combination of *L. javanica* (essential oil, aqueous and organic extract) with the conventional antifungal agents (Table 4.15), demonstrated only one antagonistic (*L. javanica* essential oil with amphotericin B against *C. albicans* with a Σ FIC of 5.34) and one synergistic interaction (*L. javanica* aqueous extract with nystatin against *C. albicans* with a Σ FIC of 0.50) (Table 4.15).

Table 4.15. MIC (μ g/ml) and Σ FIC values for the combination of *L. javanica* with the various antifungal agents, against the yeasts.

Combination	Sample type	<i>C. albicans</i> (ATCC 10231)				<i>C. neoformans</i> (ATCC 14116)			
		Ind. MIC	Combo. MIC	Σ FIC	Int.	Ind. MIC	Combo. MIC	Σ FIC	Int.
<i>L. javanica</i> + amphotericin	Aq Amp	750 1.56	500 1.56	1.67	IND	1000 0.39	130 0.39	1.13	IND
	Org Amp	1000 1.56	750 2.35	2.25	IND	380 0.39	190 0.59	2.00	IND
	EO Amp	1500 1.56	2000 6.25	5.34	ANT	380 0.39	100 0.30	1.02	IND
<i>L. javanica</i> + nystatin	Aq Nys	750 2.34	190 0.59	0.50	SYN	1000 1.56	250 0.78	0.75	ADD
	Org Nys	1000 2.34	380 1.17	0.88	ADD	380 1.56	190 0.59	0.88	ADD
	EO Nys	1500 2.34	2000 6.25	4.00	IND	380 1.56	250 0.78	1.16	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; ADD = additive interaction; IND = indifferent interaction; ANT = antagonistic interaction.

4.3.6. Combinations containing *Pelargonium sidoides*

The combination of *P. sidoides* (aqueous and organic extract) with the conventional antibiotics, demonstrated no antagonistic interactions against the Gram-positive pathogens (Table 4.16). However, 12 synergistic interactions were identified against these pathogens, which were observed against *B. cereus* (Σ FIC of 0.32 – 0.50) and *S. aureus* (Σ FIC of 0.20 –

Table 4.16. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *P. sidoides* with the various antibiotics, against the Gram-positive pathogens.

Combination	Sample type	<i>S. aureus</i> (ATCC 25923)				<i>B. cereus</i> (ATCC 11778)				<i>E. faecalis</i> (ATCC 29212)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>P. sidoides</i> + ciprofloxacin	Aq Cip	2000 0.47	750 0.24	0.89	ADD	2000 0.63	500 0.16	0.50	SYN	1000 1.25	1500 0.47	1.88	IND
	Org Cip	1500 0.47	750 0.24	1.01	IND	1500 0.63	380 0.12	0.44	SYN	2000 1.25	1000 0.32	0.76	ADD
<i>P. sidoides</i> + erythromycin	Aq Ery	2000 0.32	500 0.16	0.77	ADD	2000 0.32	250 0.08	0.39	SYN	1000 1.25	1500 0.47	1.88	IND
	Org Ery	1500 0.32	500 0.16	0.85	ADD	1500 0.32	190 0.06	0.32	SYN	2000 1.25	1500 0.47	1.13	IND
<i>P. sidoides</i> + gentamicin	Aq Gen	2000 1.88	500 0.16	0.34	SYN	2000 ≥ 2.50	1000 0.32	NA	SYN	1000 ≥ 2.50	2000 0.63	NA	IND
	Org Gen	1500 1.88	500 0.16	0.42	SYN	1500 ≥ 2.50	1000 0.32	NA	SYN	2000 ≥ 2.50	1500 0.47	NA	ADD
<i>P. sidoides</i> + penicillin G	Aq Pen	2000 ≥ 2.50	500 0.16	0.32	SYN	2000 ≥ 2.50	2000 0.63	1.25	IND	1000 ≥ 2.50	1000 0.32	1.13	IND
	Org Pen	1500 ≥ 2.50	250 0.08	0.20	SYN	1500 ≥ 2.50	750 0.24	0.60	ADD	2000 ≥ 2.50	380 0.12	0.24	SYN
<i>P. sidoides</i> + tetracycline	Aq Tet	2000 0.23	250 0.08	0.48	SYN	2000 0.16	250 0.08	0.63	ADD	1000 ≥ 2.50	1000 0.32	NA	IND
	Org Tet	1500 0.23	500 0.16	1.03	IND	1500 0.16	190 0.06	0.51	ADD	2000 ≥ 2.50	1000 0.32	NA	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction.

0.42). Only the aqueous extract of *P. sidoides*, and not the organic extract, was synergistic against *S. aureus* (Σ FIC of 0.48) when in combination with tetracycline (Table 4.16).

The combination of *P. sidoides* (aqueous and organic extract) with the conventional antibiotics, when tested against the Gram-negative pathogens (Table 4.17), demonstrated five antagonistic interactions, which includes *P. sidoides* aqueous extract with ciprofloxacin against *E. coli* (tentative antagonism), *P. sidoides* aqueous and organic extract with ciprofloxacin against *P. aeruginosa* (tentative antagonism) and lastly, *P. sidoides* aqueous and organic extract with gentamicin against *P. aeruginosa* (tentative antagonism) and three synergistic interactions, which includes *P. sidoides* organic extract with ciprofloxacin against *E. coli* (tentative synergy), *P. sidoides* aqueous and organic extract with gentamicin against *K. pneumoniae*, also showing tentative synergy (Table 4.17).

The combination of *P. sidoides* (aqueous and organic extract) with the conventional antifungals (Table 4.18), demonstrated no synergistic or antagonistic interactions against *C. albicans* and *C. neoformans*.

4.2.7. Combinations containing *Sutherlandia frutescens*

The combination of *S. frutescens* (aqueous and organic extract) with the conventional antibiotics, demonstrated no antagonism when tested against the Gram-positive pathogens (Table 4.19). Three synergistic interactions (*S. frutescens* organic extract with gentamicin against *S. aureus* (Σ FIC of 0.17), *S. frutescens* aqueous extract with tetracycline against *B. cereus* (tentative synergy) and the organic extract with penicillin G against *E. faecalis*, with a Σ FIC of 0.38) were identified against the Gram-positive pathogens tested (Table 4.19). The combination of *S. frutescens* with the conventional antibiotics, demonstrated two tentative antagonistic interactions (*S. frutescens* aqueous extract with ciprofloxacin and then gentamicin against *P. aeruginosa*), when tested against the Gram-negative pathogens. One synergistic interaction was identified, which was that of the organic extract of *S. frutescens* with ciprofloxacin against *E. coli* (Σ FIC of 0.28) (Table 4.20).

Table 4.17. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *P. sidoides* with the various antibiotics, against the Gram-negative pathogens.

Combination	Sample type	<i>E. coli</i> (ATCC 25922)				<i>K. pneumoniae</i> (ATCC 13883)				<i>P. aeruginosa</i> (ATCC 27853)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>P. sidoides</i> + ciprofloxacin	Aq Cip	≥ 8000 0.08	1000 0.32	NA	ANT	≥ 8000 0.63	3000 0.94	NA	ADD	2000 0.16	≥ 4000 ≥ 1.25	NA	ANT
	Org Cip	≥ 8000 0.08	50 0.02	NA	SYN	≥ 8000 0.63	2000 0.63	NA	IND	1500 0.16	≥ 4000 ≥ 1.25	NA	ANT
<i>P. sidoides</i> + gentamicin	Aq Gen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	1000 0.32	NA	SYN	2000 0.32	≥ 4000 ≥ 1.25	NA	ANT
	Org Gen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	500 0.16	NA	SYN	1500 0.32	≥ 4000 ≥ 1.25	NA	ANT
<i>P. sidoides</i> + tetracycline	Aq Tet	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	2000 ≥ 2.50	1000 0.32	NA	ADD
	Org Tet	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	1500 ≥ 2.50	1500 0.47	NA	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction; bold highlight = synergistic interaction.

Table 4.18. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *P. sidoides* with the various antifungal agents, against the yeasts.

Combination	Sample type	<i>C. albicans</i> (ATCC 10231)				<i>C. neoformans</i> (ATCC 14116)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>P. sidoides</i> + amphotericin	Aq	1500	750	2.00	IND	1000	130	1.13	IND
	Amp	1.56	2.34			0.39	0.39		
<i>P. sidoides</i> + nystatin	Org	2000	1500	3.76	IND	1500	190	1.63	IND
	Amp	1.56	4.69			0.39	0.59		
<i>P. sidoides</i> + nystatin	Aq	1500	500	1.00	ADD	1000	250	0.75	ADD
	Nys	2.34	1.56			1.56	0.78		
<i>P. sidoides</i> + nystatin	Org	2000	750	1.38	IND	1500	190	0.51	ADD
	Nys	2.34	2.35			1.56	0.59		

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; ADD = additive interaction; IND = indifferent interaction.

Sutherlandia frutescens is commonly ingested orally as an alcoholic tincture for the traditional treatment of UTI's (Appendix F.7), whereas ciprofloxacin is a common conventional treatment for UTI's. Therefore, testing the organic extract in combination would most closely depict the possible interactions between ciprofloxacin and *S. frutescens*, when consumed in the traditional form. The combination of *S. frutescens* (organic extract) with ciprofloxacin demonstrated a synergistic profile (ΣFIC of 0.28) (Table 4.20) against *E. coli*, which is the most common causative micro-organism of UTI's, and therefore the combination was studied further in various ratios. *Sutherlandia frutescens* can also be consumed as a herbal tea, therefore the combination of ciprofloxacin with the aqueous extract was also evaluated in various ratios, even though an indifferent interaction was identified in the ΣFIC evaluation (Table 4.20). In the varied ratio studies, most ratios for both the aqueous and organic extract combinations with ciprofloxacin were found in the additive region (Figure 4.4). Three ciprofloxacin: *S. frutescens* organic extract ratios (60:40; 50:50 and 30:70 μl) (Table 2.4) combinations were found below or on the 0.5:0.5 line, thereby demonstrating a synergistic interaction, which supports the ΣFIC evaluation (Table 4.20). Only one ciprofloxacin: *S. frutescens* organic extract ratio (10:90 μl) was found in the indifferent region, however, four of the ratios (70:30; 30:70; 20:80; 10:90 μl) for the aqueous extract combination were found in the indifferent region of the isobologram (Figure 4.4), which also supports the ΣFIC evaluation (Table 4.20).

Table 4.19. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *S. frutescens* with the various antibiotics, against the Gram-positive pathogens.

Combination	Sample type	<i>S. aureus</i> (ATCC 25923)				<i>B. cereus</i> (ATCC 11778)				<i>E. faecalis</i> (ATCC 29212)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>S. frutescens</i> + ciprofloxacin	Aq Cip	≥ 8000 0.47	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 0.63	1000 0.32	NA	ADD	≥ 8000 1.25	2000 0.63	NA	ADD
	Org Cip	2000 0.47	1000 0.32	1.18	IND	750 0.63	750 0.24	1.38	IND	4000 1.25	1000 0.32	0.51	ADD
<i>S. frutescens</i> + erythromycin	Aq Ery	≥ 8000 0.32	1500 0.47	NA	IND	≥ 8000 0.32	500 0.16	NA	ADD	≥ 8000 1.25	2000 0.63	NA	ADD
	Org Ery	2000 0.32	1000 0.32	1.53	IND	750 0.32	250 0.08	0.59	ADD	4000 1.25	1000 0.32	0.51	ADD
<i>S. frutescens</i> + gentamicin	Aq Gen	≥ 8000 1.88	2000 0.63	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Gen	2000 1.88	250 0.08	0.17	SYN	750 ≥ 2.50	2000 0.63	NA	IND	4000 ≥ 2.50	2000 0.63	NA	ADD
<i>S. frutescens</i> + penicillin G	Aq Pen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	3000 0.94	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Pen	2000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	750 ≥ 2.50	750 0.24	1.10	IND	4000 ≥ 2.50	1000 0.32	0.38	SYN
<i>S. frutescens</i> + tetracycline	Aq Tet	≥ 8000 0.23	1000 0.32	NA	IND	≥ 8000 0.16	190 0.06	NA	SYN	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Tet	2000 0.23	500 0.16	0.95	ADD	750 0.16	190 0.06	0.63	ADD	4000 ≥ 2.50	2000 0.63	NA	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction.

Table 4.20. MIC (µg/ml) and ΣFIC values for the combination of *S. frutescens* with the various antibiotics, against the Gram-negative pathogens.

Combination	Sample type	<i>E. coli</i> (ATCC 25922)				<i>K. pneumoniae</i> (ATCC 13883)				<i>P. aeruginosa</i> (ATCC 27853)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>S. frutescens</i> + ciprofloxacin	Aq Cip	≥ 8000 0.08	500 0.16	NA	IND	≥ 8000 0.63	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 0.16	2000 0.63	NA	ANT
	Org Cip	2000 0.08	50 0.02	0.28	SYN	≥ 8000 0.63	2000 0.63	NA	IND	4000 0.16	250 0.08	0.56	ADD
<i>S. frutescens</i> + gentamicin	Aq Gen	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	≥ 8000 0.32	≥ 4000 ≥ 1.25	NA	ANT
	Org Gen	2000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD	4000 0.32	380 0.23	0.84	ADD
<i>S. frutescens</i> + tetracycline	Aq Tet	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 1.25	≥ 4000 ≥ 1.25	NA	IND	≥ 8000 ≥ 2.50	≥ 4000 ≥ 1.25	NA	ADD
	Org Tet	2000 1.25	3000 0.94	2.25	IND	≥ 8000 1.25	3000 0.94	NA	IND	4000 ≥ 2.50	1500 0.47	NA	ADD

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where ≥ MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; **SYN** = synergistic interaction; *ANT* = antagonistic interaction.

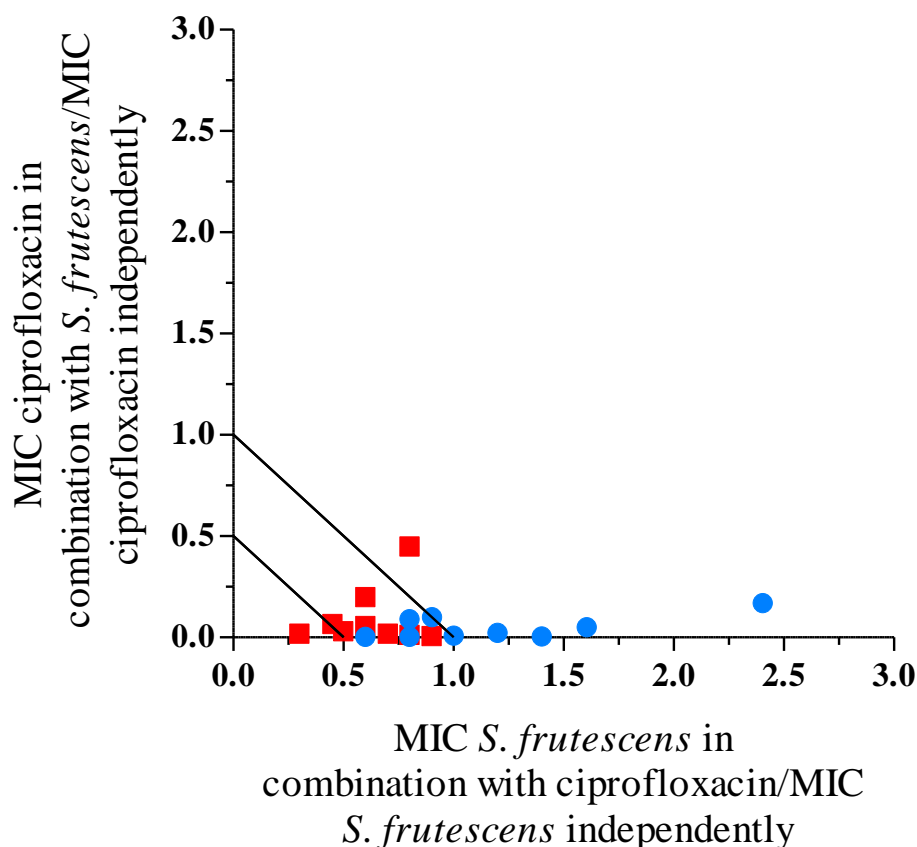


Figure 4.4. Isobologram for *S. frutescens* (● = aqueous extract; ■ = organic extract) in combination with ciprofloxacin, when tested at various ratios, against *E. coli*.

Against the yeasts (Table 4.21), the combination of *S. frutescens* (aqueous and organic extract) with the conventional antifungal agents, demonstrated a mostly antagonistic interactive profile, with five antagonistic interactions identified. These interactions were mostly seen with the aqueous extract combinations (*S. frutescens* aqueous extract with amphotericin B and nystatin against *C. albicans* and *C. neoformans*, all exhibiting tentative antagonism). One combination containing the organic extract, when in combination with amphotericin B, was found to be antagonistic against *C. neoformans* (Σ FIC of 6.76) (Table 4.21).

Table 4.21. MIC ($\mu\text{g/ml}$) and ΣFIC values for the combination of *S. frutescens* with the various antifungal agents, against the yeasts.

Combination	Sample type	<i>C. albicans</i> (ATCC 10231)				<i>C. neoformans</i> (ATCC 14116)			
		Ind. MIC	Combo. MIC	ΣFIC	Int.	Ind. MIC	Combo. MIC	ΣFIC	Int.
<i>S. frutescens</i> + amphotericin	Aq Amp	≥ 8000	≥ 4000 ≥ 12.50	NA	ANT	≥ 8000	≥ 4000 ≥ 12.50	NA	ANT
	Or Amp	3000	100 0.30	0.22	SYN	1000	750 2.35	6.76	ANT
<i>S. frutescens</i> + nystatin	Aq Nys	≥ 8000	≥ 4000 ≥ 12.50	NA	ANT	≥ 8000	≥ 4000 ≥ 12.50	NA	ANT
	Org Nys	3000	1000 3.13	1.67	IND	1000	500 1.56	1.50	IND

Shaded areas indicate the MIC values as determined for the individual samples, which have been given here as a point of reference (Ind. = individual); Combo. = MIC of agents in combination; Aq = aqueous extract; Org = organic extract; EO = essential oil; Int. = interaction classification; NA = where \geq MIC values are observed, an absolute ΣFIC value cannot be attained and are therefore referred to as a tentative interpretation; ADD = additive interaction; IND = indifferent interaction; SYN = synergistic interaction; ANT = antagonistic interaction.

4.3. General discussion and summary of results

A summary of the percentage of each interaction (synergistic, additive, indifferent and antagonistic) that was observed when 476 plant: antimicrobial sample combinations were tested in the current study has been provided in Figure 4.5. The majority of the combinations were indifferent in nature (202 of the 476 combinations tested) (Figure 4.5). Additive interactions were also prevalent (170 of the 476 combinations tested). The identification of indifferent and additive interactions alleviates some concern related to the concurrent use of these two forms of healthcare, since no advantage or disadvantage is associated with these types of interactions. However, as discussed in this chapter, notable synergistic (68 of the 476 combinations tested) and antagonistic (36 of the 476 combinations tested) interactions (Figure 4.5) were also identified, which could have an impact on treatment regimens. The data was further assessed to determine which medicinal plant and which conventional antimicrobial demonstrated the most synergistic and antagonistic interactions when placed in combination, for which the results have been recorded in Table 4.22.

Some South African medicinal plants have already been extensively investigated and have demonstrated the potential for interactions with conventional drugs. For example, *Hypoxis hemerocallidea* (African potatoe) has been shown to modulate the CYP3A4 enzyme (Mills *et al.*, 2005). Fasinu *et al.* (2013a) found that the aqueous extract of *H. hemerocallidea* has the potential to modulate other CYP450 enzymes too.

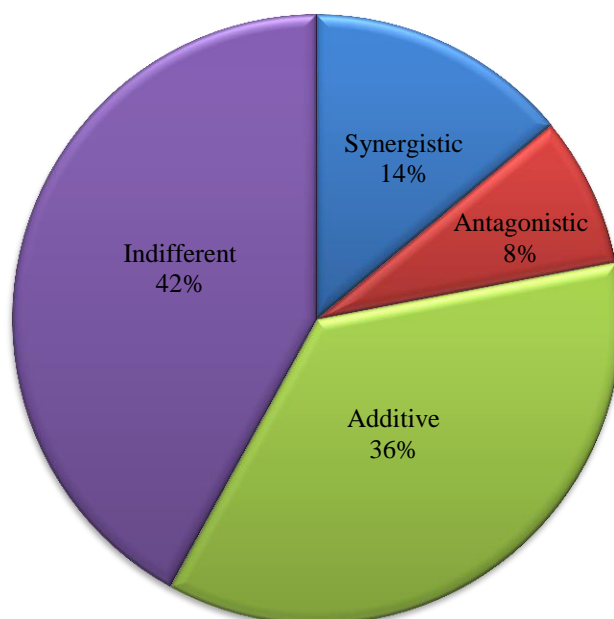


Figure 4.5. A summary of the interactions for all 476 combinations tested.

Table 4.22. A summary of the interactive profiles for each medicinal plant and conventional antimicrobial, when tested in combination.

		Synergistic (%)	Antagonistic (%)	Additive (%)	Indifferent (%)
Medicinal plants combined with antimicrobials	<i>A. betulina</i>	10.71	2.38	44.05	42.86
	<i>A. ferox</i>	5.36	16.07	48.21	30.36
	<i>A. afra</i>	9.52	8.33	33.33	48.81
	<i>A. linearis</i>	19.64	7.14	19.64	53.57
	<i>L. javanica</i>	20.24	2.38	32.14	45.24
	<i>P. sidoides</i>	26.79	8.93	30.36	33.93
	<i>S. frutescens</i>	8.93	12.50	44.64	33.93
Antimicrobials combined with medicinal plants	Ciprofloxacin	16.67	7.84	25.49	50.00
	Erythromycin	11.77	0.00	35.49	52.94
	Gentamicin	18.63	6.86	53.92	20.59
	Penicillin G	25.49	3.92	47.06	23.53
	Tetracycline	10.78	1.96	27.45	59.80
	Amphotericin B	2.94	38.24	0.00	58.82
	Nystatin	2.94	11.77	35.29	50.00

Some of the plants that have been investigated in this current study have also shown an interactive potential with conventional drugs, other than conventional antibiotics and antifungals. *Sutherlandia frutescens* in combination with antiretroviral medication has shown the ability to reduce treatment efficacy of the antiretroviral drugs (Mills *et al.*, 2005). Fasinu *et al.* (2003b) found that *S. frutescens* had the ability to delay the production of midazolam metabolites, resulting in a 40% reduction in clearance. The South African plants, *A. linearis* and *Cyclopia intermedia*, from which rooibos and honeybush tea are prepared, respectively, have been found to induce CYP3A4 enzymes, resulting in reduced efficacy of conventional drugs that are metabolised by the enzyme (Matsuda *et al.*, 2007). Another South African medicinal plant showing interactive potential, is *Harpagophytum procumbens* (devil's claw), which again has been found to have an effect on the CYP3A4 enzyme. Instead of the enzyme induction as seen with the previously mentioned examples, devil's claw inhibits the enzyme, thereby resulting in prolonged activity of conventional drugs metabolised by this enzyme, which could result in an increased risk of adverse effects and toxicity. An example is the combination of devil's claw together with warfarin, resulting in purpura (Fugh-Berman, 2000; Van den Bout-Van den Beukel *et al.*, 2006). The identification of these interactions with South African medicinal plants emphasizes the need to address the lack of information pertaining to interactions between traditional remedies and conventional drugs, particularly since so many South Africans make use of traditional medicine, where medicinal plants play a central role.

A review by Van Vuuren and Viljoen (2011), documented numerous combinations of plants with conventional antimicrobials. A summary of the results for many combination studies were given, where most often, synergy had been reported. In the review, no studies were found where conventional antimicrobials were investigated in combination with the South African medicinal plants selected for analysis in this study. This further demonstrates the lack of information pertaining to interactive South African medicinal plant: antimicrobial combinations and thus highlighting the urgent need for the scientific investigation of these combinations. In most of the studies reviewed by Van Vuuren and Viljoen (2011), the conventional antimicrobials selected for analysis in combination were ciprofloxacin, penicillin G, gentamicin, tetracycline, erythromycin, amphotericin B and nystatin. These are also the antimicrobials investigated in the current study (Table 3.1). Even though direct comparisons between the results from the review and those obtained in this current study cannot be drawn, a familiar pattern could possibly be identified, pertaining to the specific

conventional antimicrobials when in combination with plants. The review also acknowledged that synergistic effects are mostly reported in antimicrobial combination studies, and antagonistic interactions are often not reported. Antagonistic interaction documentation and reporting is of equal importance, due to the adverse effect that the interaction could have on conventional treatment outcomes. Therefore, the current study also aimed at reporting not only the synergistic interactions, but those of antagonism too.

The herb-drug interaction website, www.prescribeguide.com, provided no search results for any of the medicinal plants investigated in this study, except for *A. betulina* (buchu), which was shown to have no identified interactions, even though this current study has demonstrated its interactive potential (Table 4.1, 4.2 and 4.3). As the plants from this study are indigenous to South Africa, it is not surprising to find a lack of information pertaining to plant: drug interactions using this website. Ideally, a similar website for African traditional medicine interactions would be advantageous. The antimicrobial interactions available on the website included some of the antimicrobials investigated in this study, which are discussed.

Even though no interactions of combinations containing the selected medicinal plants in this study could be found in other literature, the selected conventional antimicrobials were extensively studied in combination with other medicinal plants and herbs. On the herb-drug interaction website, www.prescribeguide.com, ciprofloxacin was contraindicated in combination with *Berberis aquifolium*, *Berberis vulgaris* and *Hydrastis canadensis*, which are plants native to North America, Europe and the United States, respectively. Ahmad and Aqil (2006) tested ciprofloxacin in combination with crude extracts of 15 Indian medicinal plants, where the combinations showed synergistic effects when tested against enteric bacteria. Rosato *et al.* (2007) investigated the interaction between the fluoroquinolone, norfloxacin, and the essential oil of *P. graveolens* against *B. cereus*, *B. subtilis*, *E. coli* and *S. aureus*, using the agar dilution method. Synergistic interactions were identified for the combinations against the tested microbes, with a Σ FIC range of 0.37 – 0.50. Adwan *et al.* (2009) investigated the combination of the fluoroquinolone, enrofloxacin, with ethanolic plant extracts (*Rhus coriaria*, *Psidium guajava*, *Lawsonia inermis* and *Sacropoterium spinosum*) against MRSA, using the well diffusion method. Combinations with enrofloxacin showed an antagonistic interaction, where the inhibition zones were decreased when tested in combination. Since enrofloxacin and ciprofloxacin are both from the class of fluoroquinolones, similar results could be expected with both, however, this would need to be confirmed with further antimicrobial testing. Van Vuuren *et al.* (2009) evaluated the

interactions between ciprofloxacin and the essential oils of *M. alternifolia*, *T. vulgaris*, *M. piperita* and *R. officinalis*, using the micro-dilution assay, against various pathogens. In the study, a Σ FIC of < 1 was taken as synergistic and a Σ FIC of > 1 demonstrated antagonism. Therefore, using the given interpretations, the combination of ciprofloxacin with the various essential oils demonstrated mainly antagonistic profiles against *S. aureus*. However, using the interpretations as per the current study (Table 2.3), the only antagonism (Σ FIC of > 4) would be seen for the combination of *M. alternifolia* and ciprofloxacin, when tested against *S. aureus*. When the combinations were tested against *K. pneumoniae*, there was a varied interactive profile, which included synergistic, antagonistic and additive interactions. It was found that the interactions were very much dependant on the ratios in which the agents were combined and ultimately dependent on the final concentrations used. The combination of ciprofloxacin with *R. officinalis* against *K. pneumoniae* demonstrated a synergistic interaction. Adwan *et al.* (2010) studied the combination of enrofloxacin with the ethanolic extracts of *R. coriaria*, *S. spinosum* and *R. damascene* against *P. aeruginosa*, using the micro-dilution method. The combinations mostly demonstrated a reduced MIC value, thereby showing a synergistic effect against *P. aeruginosa*. Therefore, the results obtained from previous studies show that ciprofloxacin: plant containing combinations mostly demonstrated synergistic profiles, however, some antagonism was also noted. From the 102 combinations tested in the current study, where ciprofloxacin was combined with the seven selected medicinal plants and tested against the appropriate pathogens, 17 combinations were synergistic (16.67%) and eight combinations were antagonistic (7.84%). Most of the combinations containing ciprofloxacin, were found to be either additive (25.49%) or indifferent (50.00%) in nature (Table 4.22), which does not support the mostly synergistic effects seen in previous studies when ciprofloxacin was combined with other plants.

On the herb-drug interaction website, www.prescribeguide.com, erythromycin was contraindicated in combination with *H. perforatum* (St. John's wort), *H. canadensis* (goldenseal) and *Citrus paradisi* (grapefruit) juice. Darwish *et al.* (2002) tested the combination of erythromycin with ethanolic extracts of 19 Jordanian plants against both sensitive and resistant strains of *S. aureus*. It was found that the antimicrobial activity of erythromycin was enhanced when in combination with the extracts; however, the effect was more pronounced against the sensitive strain of *S. aureus*, rather than the resistant strain. Oluwatuyi *et al.* (2004) investigated the combination of *R. officinalis* compounds, carsonic acid and carnosol, with erythromycin against a resistant strain of *S. aureus*, where a 32- and

16-fold antimicrobial potentiation was noted for each combination, respectively. Adikwu *et al.* (2010) found that erythromycin, when in combination with the methanolic extract of *Euphorbia hirta* showed synergy against *S. aureus*. In a varied ratio study, seven of the nine tested ratios showed a synergistic interaction, in the checkerboard method. Indifference was found for the other two ratios (5:5; 4:6). Soutu de Oliveira *et al.* (2011) tested erythromycin in combination with the ethanolic extract of the peel of *Mangifera indica* against *S. aureus*, where the extract was found to reduce the MIC of erythromycin 4-fold, thereby demonstrating a strong synergistic interaction. Therefore, the results found in previous studies demonstrate that erythromycin: plant containing combinations are mostly synergistic in nature. From the 51 combinations tested in this study where erythromycin was combined with the seven medicinal plants and tested against the appropriate pathogens, only six combinations were found to synergistic (11.77%). No antagonism was identified for any combinations containing erythromycin (Table 4.22), which is supported by the previously mentioned studies. Erythromycin was thus the antibiotic that demonstrated the least antagonism when combined with the medicinal plants. The plant: erythromycin combinations tested in this study demonstrated mostly indifferent interactive profiles (52.94%) (Table 4.22).

Darwish *et al.* (2002) found that when gentamicin was combined with ethanolic extracts 19 Jordanian plants, an enhanced antimicrobial activity was observed when tested against *S. aureus*. Braga *et al.* (2005) tested gentamicin in combination with the methanolic extract of *Punia granatum* (pomegranate) against 30 clinical isolates of MRSA and methicillin-sensitive *S. aureus* (MSSA), and a 38.10% synergy was observed for the combination against the 30 isolates. Adwan *et al.* (2009) studied the combination of gentamicin with plant extracts (*R. coriaria*, *P. guajava*, *L. inermis* and *S. spinosum*) against MRSA, using the agar diffusion method. The combinations mostly resulted in an increased zone of inhibition, thereby demonstrating a synergistic interaction. However, an antagonistic interaction between gentamicin and *P. guajava* was identified. Toroglu (2011) tested gentamicin in combination with the essential oils of some herbs (*R. officinalis*, *M. piperita*, *Coriandrum sativum*, *Micromeria fruticosa* and *Cuminum cyminum*), against 13 microbes. The only essential oil that did not demonstrate a synergistic effect when in combination with gentamicin was *C. sativum*. All other herb essential oils demonstrated synergistic interactions, with the combination of *M. fruticosa* and gentamicin displaying synergy against all 13 tested pathogens. Therefore, the results observed from the previous studies pertaining to

combinations of gentamicin with plants, demonstrated a mostly synergistic profile. From the 102 combinations tested in this study, where gentamicin was combined with the seven selected medicinal plants and tested against the appropriate pathogens, only 19 combinations were found to be synergistic (18.63%), whilst seven combinations were found to be antagonistic (6.86%). The majority of the plant: gentamicin combinations tested demonstrated an additive profile (53.92%) (Table 4.22).

Darwish *et al.* (2002) investigated the combination of penicillin with ethanolic extracts of 19 Jordanian plants against both sensitive and resistant strains of *S. aureus* and found that the antimicrobial activity of penicillin was enhanced when in combination, with the effect more pronounced against the sensitive strain rather than the resistant strain. Braga *et al.* (2005) tested ampicillin, a derivative of penicillin, in combination with the methanolic extract of *P. granatum* against 30 isolates of MRSA and MSSA, and synergistic interactions were found against 71.40% of the isolates. Al-hebshi *et al.* (2006) investigated penicillin G in combination with aqueous extract of *C. edulis* and found a synergistic interaction, since the MIC value of the conventional antimicrobial was reduced when tested in combination, against 33 resistant oral pathogens. From the 51 combinations tested in this current study, where penicillin G was combined with the seven medicinal plants and tested against the appropriate pathogens, 13 were found to be synergistic (25.49%), whilst two combinations were antagonistic (3.92%). Penicillin G was thus the antibiotic which demonstrated the most synergistic interactions with the selected medicinal plants. The majority of the plant: penicillin G combinations tested demonstrated an additive profile (47.06%), as observed with many other conventional antimicrobial combinations in this study (Table 4.22).

Darwish *et al.* (2002) tested tetracycline with ethanolic extracts of Jordanian plants and found that there was an increased antimicrobial activity against both resistant and sensitive strains of *S. aureus*, with the effect being more pronounced for the resistant strain, as opposed to the sensitive strain. Oluwatuyi *et al.* (2004) investigated the combination of *R. officinalis* derived compounds, carsonic acid and carnosol, with tetracycline, where a 2- and 4-fold antimicrobial potentiation was observed against a resistant strain of *S. aureus*, respectively. Braga *et al.* (2005) tested tetracycline in combination with the methanolic extract of *P. granatum* (pomegranate) against 30 isolates of MRSA and MSSA, and synergy was noted against 70.00% of the 30 tested isolates. Ahmad and Aqil (2006), studied tetracycline in combination with crude extracts of 15 Indian medicinal plants, where the combinations showed synergistic effects when tested against enteric bacteria. Al-hebshi *et al.* (2006) found that tetracycline in

combination with the aqueous extract of *C. edulis* displayed a synergistic interaction, since the MIC value of tetracycline against 33 resistant oral pathogens was reduced when in combination. Adwan *et al.* (2009) studied the combination of oxytetracycline with the ethanolic plant extracts of *R. coriaria*, *P. guajava*, *L. inermis* and *S. spinosum* against MRSA, using the well diffusion method. Mostly synergistic interactions were identified for the combination, due to the increased zones of inhibition of the combination. Adwan *et al.* (2010) also investigated the combination of oxytetracycline with the ethanolic extracts of *R. coriaria*, *S. spinosum* and *R. damascene* against *P. aeruginosa*, using the micro-dilution assay. Synergistic interactions were most often seen, with reduced MIC values for the combination. Soutu de Oliveira *et al.* (2011) tested tetracycline in combination with the ethanolic extracts of the peel of *M. indica* against *S. aureus*. The extract was found to reduce the MIC of tetracycline four-fold, demonstrating a strong synergistic combination. Therefore, from these previous studies of tetracycline in combination with natural products, mostly synergistic interactions were identified and reported. From the 102 combinations tested in this current study, where tetracycline was combined with the seven medicinal plant samples and tested against the appropriate pathogens, only 11 combinations were found to be synergistic (10.78%) and only two combinations demonstrated antagonism (1.96%). The majority of the plant: tetracycline combinations were found to be indifferent (59.80%) in nature (Table 4.22).

On the herb-drug interaction website, www.prescribeguide.com, amphotericin B was indicated as having a synergistic interaction with curcumin against *Candida* species, which was supported in a study by Sharma *et al.* (2010). Shin (2003) tested amphotericin B in combination with the essential oil of *P. graveolens* and its main components and found additive effects against *Aspergillus niger* and *A. flavus*, with a Σ FIC range of 0.52 to 1.00. Rosato *et al.* (2008) tested amphotericin B in combination with the essential oils of *M. alternifolia*, *Origanum vulgare* and *P. graveolens*, against various *Candida* species, using the micro-dilution and diffusion assays. Synergistic interactions were identified, with the combination of *P. graveolens* and amphotericin B showing the most synergy. Van Vuuren *et al.* (2009) evaluated the interactions between amphotericin B and the essential oils of *M. alternifolia*, *T. vulgaris*, *M. piperita* and *R. officinalis*, using the micro-dilution assay against *C. albicans*. In the study, antagonism was demonstrated for Σ FIC values of > 1 and therefore most of the combinations of the four essential oils with amphotericin B showed antagonism against *C. albicans*, in various ratios. However, for this current study, interactions were

classified as antagonistic for Σ FIC values > 4 (Table 2.3), and using this interaction interpretation, none of the combinations tested by Van Vuuren *et al.* (2009) were antagonistic against *C. albicans*. Therefore, from these latter combination studies with amphotericin B, it was found that interactive profiles varied between synergistic, additive and antagonistic interactions when tested against yeasts or moulds. In the current study, a large proportion (13 of the 34 amphotericin B containing combinations) demonstrated antagonistic profiles (38.24%) when tested against *C. albicans* and *C. neoformans*. Amphotericin B was thus the antimicrobial which demonstrated the most antagonistic interactions when combined with the seven selected medicinal plants. Only one of the combinations containing amphotericin B was found to be synergistic (2.94%). Therefore, amphotericin B was the antimicrobial which demonstrated the least synergistic interactions when combined with the medicinal plants. The majority of the plant: amphotericin B combinations were found to be indifferent in nature (58.82%) (Table 4.22).

Rosato *et al.* (2009) evaluated the combination of nystatin with the essential oils of *M. alternifolia*, *O. vulgare* and *P. graveolens* against five different *Candida* strains, using the micro-dilution assay. Synergistic interactions were identified when combined with *O. vulgare* and *P. graveolens*, however, only additive effects were identified for the combination of *M. alternifolia* and nystatin. The combination of *O. vulgare* and nystatin demonstrated the most synergy against the strains of the yeast, with a Σ FIC range of 0.11 to 0.17. Therefore, from the previous study of combinations of nystatin with plants, it was found that only synergistic additive interactions were noted. In the current study, the majority of the combinations demonstrated additive interactions (35.29%) and more antagonistic interactions (11.77%) were noted for the nystatin combinations, than synergistic interactions (2.94%). Nystatin was thus, along with amphotericin B, the antimicrobial demonstrating the least synergistic interactions (Table 4.22).

4.4. Conclusions

- Of the 476 conventional antimicrobial: medicinal plant combinations studied, 14% of all the combinations were synergistic, 8% were antagonistic, 36% were additive and 42% were indifferent in nature.
- *Agathosma betulina* aqueous and organic extract combined with ciprofloxacin against *E. coli* provided tentative synergistic interactions, which should be further

investigated for *in vivo* effects, since the concurrent use of the traditional and conventional form of UTI treatment could have a considerably positive impact on the treatment outcome.

- *Artemisia afra* aqueous extract in combination with ciprofloxacin provided a notable antagonistic interaction (Σ FIC of 8.55) when tested against *E. coli*. Unexpectedly, the organic extract and essential oil demonstrated a synergistic interaction (Σ FIC of 0.27 for both combinations).
- The combination of *A. linearis* (aqueous and organic extract) and penicillin G is a notable combination, since a mostly synergistic profile was seen against all three of the Gram-positive pathogens tested.
- *Sutherlandia frutescens* organic extract with ciprofloxacin provided a notable synergistic interaction (Σ FIC of 0.28) against *E. coli*, which again should be further investigated for *in vivo* effects, since the combination could have an impact on UTI treatment.
- When *P. sidoides* was combined with all seven of the conventional antimicrobials and tested against the various pathogens, synergistic interactions (26.79%) were frequently encountered, more so than seen with any of the other medicinal plants in combination.
- *Aloe ferox* combinations with the conventional antimicrobials demonstrated antagonistic interactions (16.07%) frequently, more so than seen with any of the other combinations.
- The antimicrobial, penicillin G, in combination with the selected plants and when tested against the Gram-positive pathogens, frequently demonstrated a synergistic interactive profile (25.49%), more so than any other antimicrobial when in combination with the plant samples.

- The antimicrobial, amphotericin B, in combination with the selected plants and when tested against the two yeasts, frequently demonstrated an antagonistic interactive profile (38.24%), more so than any other antimicrobial when combined with the plant samples.

Chapter 5

Toxicity analysis of individual samples and some notable combinations

5.1. Introduction

Natural medicinal products, often prepared from medicinal plants, have gained popularity due to the belief that they are safe and free of toxicity (Bateman *et al.*, 1998). This belief is, however, not always true, as demonstrated by the many poisonous plants in existence. Poisonous plants are commonly found, even in ones' garden. These plants form a very important part of the indigenous flora of South Africa and some are known to exert toxicity as a defence mechanism against plant-eating animals, to either deter them or to kill them. Some plants that are most commonly associated with human poisoning include *Melia azedarach* (syringa berries), *Zantedeschia aethiopica* (arum lily leaves), *Solanum pseudocapsicum* (Jerusalem cherry fruits) and *Ricinus communis* (castor oil seeds) (Van Wyk *et al.*, 2002).

It is said that in order for both medicinal plants and conventional medicine to have a therapeutic effect, they require biological activity. This allows for toxicity to occur when these agents are taken in large doses (Van Wyk *et al.*, 2002). In traditional medicine, it has been acknowledged that very little is known of the difference between a therapeutic and lethal dose. A common medicinal plant that is therapeutic at low doses, but lethal at high doses is *Digitalis purpurea* (digitalis), from which the cardiac glycoside, digoxin, is derived (Botha and Penrith, 2008). An interesting finding is that poisoning due to plants is a less common occurrence than poisoning related to the misuse of orthodox medicine (Van Wyk *et al.*, 2002); however, this may be due to under reporting. A study by Du Plooy *et al.* (2001), found that from all the poisoning cases reported at Ga-Rankuwa Hospital in one particular year, 4.7% were attributed to traditional medicine poisoning, with 6.4% due to orthodox medicine.

Traditional remedies are not subject to the in-depth scrutiny of safety and efficacy studies, as with conventional drugs, since registration of traditional remedies with the Medicines Control Council has not yet been enforced in South Africa (Padayachee, 2011). Therefore, very little scientific information is usually available on the safety and toxicity profiles of many medicinal plants. The safety of plants for human consumption in traditional healing practices was discovered through a trial and error process and the use of medicinal plants for hundreds of years has resulted in the assumption that these plants are safe and free of toxicity. It has been acknowledged, however, that subtle and chronic forms of toxicity may not have been identified by previous generations. This notion is worrisome, since safety should be of utmost importance, especially since such a large proportion of the population makes use of medicinal plants. Many safety concerns are related to natural product use. Some of these concerns are extrinsic, such as the exposure of plant material to contaminants, or intrinsic, such as a plant naturally containing toxic substances. A further safety concern is the possibility for interactions between natural products and synthetic, conventional drugs (Tomlinson and Akerele, 1998). The lack of evidence pertaining to the toxicological profiles of medicinal plants, when used individually, as well as in combination with conventional medicine is a major problem, which needs to be addressed (Fennel *et al.*, 2004). The effects of combinations between medicinal plants and conventional drugs has not been extensively investigated, however, a few assumptions have been made. In reviews by Aiyegoro and Okoh (2009), as well as by Van Vuuren and Viljoen (2011) it has been noted that combinations may have the ability to reduce the possibility of toxicity, due to reduced dose administration. Therefore, the aim of this chapter was to evaluate the toxicity of the individual plant and antimicrobial samples, as well as some notable plant: antimicrobial combinations that were identified.

5.2. Results and discussion

5.2.1. Toxicity analysis of individual plant and antimicrobial samples

The individual plant samples and antimicrobials were investigated for toxicity, using both the BSLA and the MTT assay and the results for both assays have been recorded in Table 5.1. Increased formazan production was noted for some of the samples, which could have been either due to increased mitochondrial activity or increased cell numbers.

Table 5.1. Mortality \pm S.D (%) and cell death \pm S.D (%) results (n=6) of individual samples.

	Sample	BSLA study		MTT study
		Mortality \pm S.D (%) ^a		Cell death \pm S.D (%) ^b
		After 24 hrs:	After 48 hrs:	After 48 hrs:
Antimicrobials	Ciprofloxacin	0.00	0.00	0.10 \pm 0.01
	Erythromycin	0.00	0.00	0.10 \pm 0.01
	Gentamicin	1.12 \pm 0.58	8.99 \pm 0.33	0.10 \pm 0.01
	Penicillin G	0.00	0.00	0.10 \pm 0.01
	Tetracycline	0.00	6.67 \pm 1.16	0.10 \pm 0.01
	Amphotericin B	0.00	0.00	5.93 \pm 3.41
	Nystatin	0.00	0.00	0.10 \pm 0.01
Essential oils	<i>A. betulina</i>	100.00 \pm 0.00	100.00 \pm 0.00	35.90 \pm 6.29
	<i>A. afra</i>	0.00	1.39 \pm 0.58	31.72 \pm 4.64
	<i>L. javanica</i>	0.58 \pm 0.52	1.17 \pm 0.71	0.10 \pm 0.01
Aqueous extracts	<i>A. betulina</i>	0.00	0.00	0.10 \pm 0.01
	<i>A. ferox</i>	0.00	0.00	0.10 \pm 0.01
	<i>A. afra</i>	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i>	0.00	0.00	0.10 \pm 0.01
	<i>L. javanica</i>	0.00	1.43 \pm 0.58	0.10 \pm 0.01
	<i>P. sidoides</i>	0.00	3.45 \pm 0.58	0.10 \pm 0.01
	<i>S. frutescens</i>	0.00	0.00	0.10 \pm 0.01
Organic extracts	<i>A. betulina</i>	0.00	0.00	0.10 \pm 0.01
	<i>A. ferox</i>	0.00	0.00	0.10 \pm 0.01
	<i>A. afra</i>	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i>	0.00	0.00	0.10 \pm 0.01
	<i>L. javanica</i>	0.00	70.13 \pm 5.29	0.10 \pm 0.01
	<i>P. sidoides</i>	0.00	0.00	0.10 \pm 0.01
	<i>S. frutescens</i>	13.46 \pm 0.58	82.69 \pm 4.51	0.10 \pm 0.01
Controls	Quinine	0.00 ^a	0.00 ^a	71.38 \pm 4.73 ^a
		0.00 ^b	11.76 \pm 1.00 ^b	0.10 \pm 0.01 ^b
	Camptothecin	0.00 ^a	2.08 \pm 0.58 ^a	76.07 \pm 2.94 ^a
		30.00 \pm 2.00 ^b	100.00 \pm 0.00 ^b	0.10 \pm 0.01 ^b
	Potassium dichromate	100.00 \pm 0.00 ^c		NT

^a = Tested at a concentration of 1 mg/ml; ^b = Tested at a concentration of 100 μ g/ml; ^c = Tested at a concentration of 1.60 mg/ml; S.D. = standard deviation; NT = control not tested in assay.

5.2.1.1. Controls

In the BSLA, the negative control consisted of artificial salt water, with no addition of sample, and demonstrated a 0.00% mortality in every plate. The positive control consisted of potassium dichromate (1.60 mg/ml), which resulted in 100% mortality of the brine-shrimp. The highly toxic nature of the inorganic chemical reagent has also been noted in rabbits and rodents, where concentrations as low as 14 mg/kg have a 50% fatality rate (Sigma-Aldrich MSDS, 2012; Tikare *et al.*, 2012). Whilst in humans it has been reported to cause allergic dermatitis, carcinogenesis and ocular toxicity (Merck Manual, 2006; Sigma-Aldrich MSDS, 2012).

Quinine and camptothecin, used as the positive controls in the MTT assay, were also tested in the BSLA for toxicity, for comparison with results seen in the MTT assay (Table 5.1). Quinine did not demonstrate toxicity; however, camptothecin demonstrated a 100% mortality after 48 hours, when tested at 1 mg/ml. This is an expected result since camptothecin is a commonly used chemotherapeutic agent inhibiting cell growth by inhibiting the deoxyribonucleic acid (DNA) topoisomerase I enzyme (Merck Manual, 2006).

In the MTT assay, before plating out of the cells, a cell viability of 95% or greater was always ensured by using the Trypan blue exclusion assay to account for the blue-stained non-viable cells. The sample-free cell suspension control always exhibited a cellular viability of 100%, ensuring the cells were grown under optimal conditions. The positive controls used included quinine (100 µg/ml and 1 mg/ml) and camptothecin (100 µg/ml and 1 mg/ml). Both quinine and camptothecin demonstrated cell death ($73.38 \pm 4.73\%$ and $76.07 \pm 2.94\%$, respectively) at 1 mg/ml (Table 5.1). Therefore, some toxicity was demonstrated at 1 mg/ml only. Flaks (1978) found that in the early stages of chronic 0.1% quinine sulphate administration in drinking water, minimal tubular cell necrosis occurred in the renal cortex of rats. Worden *et al.* (1987) found that 100 – 120 mg of quinine hydrochloride consumed in tonic water on a daily basis for 14 days showed no adverse effect in humans. Colley *et al.* (1989) discovered the kidney as a target organ for quinine hydrochloride induced toxicity, since there were increased levels of plasma urea and inorganic phosphorus in rats. It was also found that there was an increase in kidney weight in male rats when 85 – 120 mg/kg quinine hydrochloride was administered for a three month period. However, no morphological changes were identified.

5.2.1.2. Conventional antimicrobials

5.2.1.2.1. Ciprofloxacin

Ciprofloxacin when tested individually in the BSLA, demonstrated no toxicity, with a mortality of 0.00% after 24 and 48 hours. In the MTT assay, ciprofloxacin also demonstrated no toxicity, with a cellular viability greater than 100% at a concentration of 100 µg/ml after 48 hours of continuous exposure (Table 5.1).

In the literature, ciprofloxacin has been found to rarely demonstrate toxic effects, however, the most common adverse reactions included GI effects, central nervous system (CNS) toxicity, along with damaging effects on cartilage and cardiovascular (CV) toxicity by prolonging the QT interval (Merck Manual, 2006; Appendix G.1). Therefore, no renal toxicity has been documented, which is supported by the findings in this study. Similarly, ciprofloxacin has been shown to not affect Caco-2 colon and bronchial lung epithelial cells *in vitro* (Griffiths *et al.*, 1993; Ong *et al.*, 2013), indicating that ciprofloxacin is relatively non-toxic to certain cell types.

5.2.1.2.2. Erythromycin

Erythromycin when tested individually in the BSLA demonstrated no toxicity, with a mortality of 0.00% after 24 and 48 hours. In the MTT assay, erythromycin also demonstrated no toxic effects on the human kidney epithelial cells (Table 5.1). Similarly, erythromycin was considered non-toxic to primary hepatocytes or other epithelial cell lines (Viluksela *et al.*, 1996; Inoue *et al.*, 2004).

In literature, erythromycin has been found to cause GI effects, cardiotoxicity, along with hepatotoxicity and auditory toxicity (Merck Manual, 2006). No renal toxicity has been reported with erythromycin use, which is supported by the findings in the current study.

5.2.1.2.3. Gentamicin

Gentamicin when tested individually in the BSLA, showed a mortality of $1.12 \pm 0.58\%$ and $8.99 \pm 0.33\%$ after 24 and 48 hours, respectively. However, it is still considered non-toxic, since the mortality is well below 50%. In the MTT assay, gentamicin did not affect cellular viability of the human kidney epithelial cells, therefore showing no toxicity (Table 5.1). These findings contrast with those by Alfonso *et al.* (1990), who observed that 250 µg/ml and 1 mg/ml gentamicin increased cellular granularity in rabbit corneal epithelial cells after 48

hours of exposure. All aminoglycoside antibiotics are generally accepted to accumulate in renal proximal tubule cells from the luminal surface and show toxic effects on the cells (Kiyomiya *et al.*, 2000), which are usually reversible, compared to auditory toxicity which can often be irreversible. The risk for toxicity is increased with larger doses, longer durations of therapy and more frequent dosing, all resulting in higher blood levels (Merck Manual, 2006). The renal toxicity of gentamicin was not supported by the *in vitro* tests conducted in this study (Table 5.1) due to the concentrations that were tested and the duration of testing.

5.2.1.2.4. Penicillin G

Penicillin G was tested individually for toxicity in the BSLA and demonstrated a 0.00% mortality after 24 and 48 hours. In the MTT assay, penicillin demonstrated a cellular viability of $107.15 \pm 9.01\%$, thereby demonstrating no toxicity (Table 5.1). This favourable safety profile was also observed in mice where a LC_{50} value was reported to be in the range of 2 g/kg (Doerr *et al.*, 1980).

Penicillin most commonly causes hypersensitivity effects, including anaphylaxis. The antibiotic is known to be neurotoxic at high doses and all penicillins have been found to cause nephritis and sometimes severe nephrotoxicity, along with some toxic effects on the blood components (Merck Manual, 2006; Granowitz and Brown, 2008).

5.2.1.2.5. Tetracycline

As with the other antibiotics, the individual tetracycline did not lethal to the brine-shrimp after 24 hours of continuous exposure, however, after 48 hours, a mortality of $6.67 \pm 1.16\%$ was observed, which is still considered non-toxic. Similarly, in the MTT assay, tetracycline did not adversely affect the human kidney epithelial cell line (Table 5.1). Although it is known that 100 – 500 µg/ml of several tetracyclines, of which doxycycline showed the strongest effect, led to a significant inhibition of cytoplasmic protein synthesis without affecting the rate of glycolysis and respiration or the energy charge in epithelial cells (De Jonge, 1973). This effect would not have been observed over the 48 hours in the MTT assay. It has also been reported that high concentrations of oxytetracycline caused severe damage of structure and function of the small intestinal epithelium in rats (Babich and Tipton, 2002).

Tetracyclines have been found to demonstrate hepatotoxicity, GI effects and damaging effects on bone and teeth in children (Merck Manual, 2006). No renal toxicity has been reported, and therefore the absence of toxicity in this current study supports this.

5.2.1.2.6. Amphotericin B

Amphotericin B showed no significant toxicity in either the BSLA or MTT assay. In the latter assay, amphotericin B killed $5.93 \pm 3.41\%$ of the human kidney epithelial cells (Table 5.1).

Amphotericin B has been known to have a very high level of toxicity with renal toxicity the most common toxic effect (Merck Manual, 2006). This toxicity was not observed in this current study in the MTT assay, due to the concentrations and durations of testing. The cellular viability observed for amphotericin B was, however, the lowest seen in comparison with the other antimicrobials and it was the only antimicrobial that demonstrated a cellular viability below 100%. Amphotericin B is known to significantly reduce trans-epithelial potential difference in human nasal epithelial cells, but not by generalized cellular toxicity as determined by mitochondrial activity, but was related to inhibitory effects of amphotericin B on ion transport proteins (Jornot *et al.*, 2005).

5.2.1.2.7. Nystatin

Nystatin showed no toxic effect when tested individually in the BSLA and MTT assay over 24 and/or 48 hours of continuous exposure (Table 5.1).

Nystatin is known to rarely demonstrate any potential for toxicity, as this agent is usually for topical application to mucous membranes. However, some hypersensitivity reactions have occurred, resulting in a facial rash, swelling and bronchospasm. Nystatin, even if orally ingested for a GI infection, shows negligible absorption from the GIT into the bloodstream. However, if large doses were to enter circulation, cardiac toxicity could occur (Merck Manual, 2006; Katzung *et al.*, 2009). Nystatin is known to increase cell permeability by disrupting ion transport and to affect glucose transport in human airway epithelial cells at a concentration of 50 $\mu\text{g/ml}$ (Ito *et al.*, 2001). Nystatin has been shown to cause cellular toxicity towards J774 macrophages and lyse red blood cells, and at a concentration of 100 fold lower than tested in this study, did not affect the viability of the RAW 264.7 mouse macrophage cell line (Tzimogianni *et al.*, 1989). The possibility for nystatin causing toxicity against human kidney epithelial cells is highly unlikely, since nystatin has negligible absorption into the systemic circulation, thus passing the renal excretory pathway (SAMF, 2012). Intravenously administered nystatin is known to possess toxicity and as such is now only administered topically, where the drug penetrates the surface layer (epidermis), but does not transverse intact skin to the blood stream (Sheppard and Lampiris, 2012; LIFE, 2013).

Topical adverse effects have been reported to include hypersensitivity, skin irritation and pruritis (Sheppard and Lampiris, 2012).

5.2.1.3. Medicinal plants

The plants selected for this study have been found to be relatively safe and free of toxicity at therapeutic doses according to literature. The book “Poisonous Plants of South Africa” by Van Wyk *et al.* (2002) is a comprehensive review of all poisonous plants in the country and the only plant from the seven selected for analysis in this study to make an appearance in the book is *L. javanica*.

5.2.1.3.1. *Agathosma betulina*

Agathosma betulina, when tested individually in the BSLA demonstrated no toxicity for the aqueous and organic extract, however, the essential oil induced $100.00 \pm 0.00\%$ mortality of the brine-shrimp, within the first 24 hours of exposure (Table 5.1). This was an irreversible effect as after 48 hours, there was still no movement observed and therefore the essential oil did not act as a reversible neuromuscular blocker, which could have given the appearance that the brine-shrimp had died within the first 24 hours. Upon testing a number of dilutions of the essential oil in the BSLA, an LC_{50} value of 0.31 ± 0.03 mg/ml was obtained from a log-sigmoid dose response curve. For the BSLA, results were interpreted and classifications were all in accordance with the study by Bussmann *et al.* (2011). Therefore, according to the classifications of toxicity for the BSLA, the essential oil of *A. betulina* demonstrated moderate toxicity (Section 2.8.1.3). Essential oils are usually used for inhalation or external application only. Oral ingestion needs to be very closely monitored due to the high possibility of toxicity, where the highly lipophilic nature of the oil could disrupt cell membranes (Wormwood, 1990).

Similarly, in the MTT assay, the aqueous and organic extract of *A. betulina* did not inhibit cellular growth (Table 5.1). However, as in the BSLA, the essential oil did demonstrate potential for toxicity toward the human kidney epithelial cells, with $35.90 \pm 6.21\%$ cell death being observed. In the MTT assay, a percentage cellular viability less than 50% was considered toxic in nature at 100 μ g/ml (Naidoo, 2013), therefore, an IC_{50} was not calculated for the essential oil of *A. betulina*, since cellular viability was still greater than 50% (Table 5.1).

In previous studies, *A. betulina* demonstrated no toxic effect on human kidney epithelial cells ($IC_{50} > 100 \mu\text{g/ml}$). The only adverse effects seen with consumption of the plant have been allergic reactions, which is attributed to the high eugenol content of the plant (Moolla, 2005). In a review by Cowan (1999), it was found that the essential oil of *A. betulina* demonstrated a relative toxicity of 2.0, where 0.0 was considered very safe and 3.0 was considered very toxic. Therefore, the identification of moderate toxicity in the current study supports the findings in the earlier review.

5.2.1.3.2. *Aloe ferox*

The aqueous and organic extract of *Aloe ferox* when tested individually in both the BSLA and MTT assays demonstrated no toxicity at 1 mg/ml and 100 $\mu\text{g/ml}$ after 48 hours of continuous exposure, respectively (Table 5.1).

There is no evidence of cytotoxicity when *A. ferox* is consumed in low doses (Kambizi and Afolayan, 2008; Wintola *et al.*, 2011), however, in large quantities, toxicity has been reported. The toxic effects include joint weakness and partial paralysis. Overdoses can also lead to nephritis, gastritis and pelvic congestion (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996). Since the extracts were tested at low concentrations (100 $\mu\text{g/ml}$ in the MTT assay), effects on the human kidney epithelial cells were not found to be evident. Concentrations greater than 100 $\mu\text{g/ml}$ could not be tested due to the interference of the plant sample colour with absorbance readings.

5.2.1.3.3. *Artemisia afra*

Artemisia afra essential oil, aqueous and organic extracts were tested individually in the BSLA. The essential oil did not affect the brine-shrimp within the first 24 hours of exposure, with only a low $1.39 \pm 0.58\%$ mortality occurring after 48 hours. Both the aqueous and organic extracts of *A. afra*, when tested individually, had no inhibitory effects on brine-shrimp viability. As such, the essential oil, aqueous and organic extract of *A. afra* were all non-toxic in the BSLA. This was also true for the aqueous and organic extract of *A. afra*, when tested individually in the MTT assay, but the essential oil demonstrated a potential for toxicity, with a cellular viability of $68.28 \pm 4.64\%$ at 100 $\mu\text{g/ml}$ (Table 5.1). This lack of toxicity has also been demonstrated following acute intra-peritoneal and oral doses, where the LC_{50} values were 2.45 and 8.96 g/kg, respectively. In addition, when administered acutely, it

has low chronic toxicity potential, and in high doses, was shown to have a hepatoprotective effect (Mukinda and Syce, 2007).

It has been acknowledged in previous studies that toxicity only occurs when large doses of *A. afra* are consumed. The toxic effects include pulmonary oedema, haemorrhagic nephritis and degenerative liver changes. The most commonly known toxic effects are the CNS effects including hallucinations and confusion, which are proposed to be due to the thujone content of the plant (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996; Mukinda and Syce, 2007; Van Wyk *et al.*, 2009). It has been acknowledged by Dube (2006), that information on the safety and efficacy of *A. afra* derived from clinical trials is lacking or under reported. Therefore, there is a definite need for further *in vivo* studies and better reporting from clinical trials and doctors/healers.

5.2.1.3.4. *Aspalathus linearis*

In the current study, when tested individually, the aqueous and organic extract of *A. linearis* were found to be non-toxic in both the BSLA and MTT assay (Table 5.1).

The lack of toxicity for *A. linearis* is supported in a previous study, where no toxic effects on the kidneys or liver of rats were displayed with chronic consumption of *A. linearis*, where the tea was provided as the sole drinking fluid for the rats over a 10 week period (Marnewick *et al.*, 2003). Later, Marnewick *et al.* (2011) also found that fermented rooibos had no adverse effects on kidney function in 83 human male and female participants, when six cups of rooibos tea were consumed per day for six weeks. Even though some of the kidney function indicators were increased after the test period, all were still within reference ranges, therefore supporting the safety of short term rooibos consumption.

5.2.1.3.5. *Lippia javanica*

In the BSLA, the essential oil and aqueous extract of *L. javanica* displayed minimal inhibitory effects on brine-shrimp viability; which was supported by the lack of inhibition of cellular mitochondria activity in the MTT assay. However, after 48 hours the toxic effects of the organic extract was greatly potentiated in the BSLA with the mortality rate increasing 49-fold, where a LC_{50} of 0.51 ± 0.03 mg/ml was obtained (Table 5.1). According to the classification of toxicity by Bussmann *et al.* (2011) based on the LC_{50} value obtained in this study, *L. javanica* organic extract is considered low or weak in toxicity (Section 2.8.1.3). A toxic profile was also observed in mice when the aqueous extracts of *L. javanica* leaves were

evaluated for toxicity. While evidence suggested that *L. javanica* has low mammalian toxicity, within 48 hours all mice fed with 12.5 – 37.5% (v/v) extract were lethargic, and the overall mortality was 37.5% (Madzimore *et al.*, 2011). Thus, despite the apparent safety, the aqueous extracts of *L. javanica* leaves may have health implications on humans and animals if consumed at very high doses. But the therapeutic range needs to be determined in pharmacokinetic studies to better advise patients and traditional healers on the quantities safe for consumption. In literature, photosensitivity is commonly reported as an adverse effect and is seen when this plant is consumed in large quantities (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996). The triterpenoids found in the *Lippia* species are toxic compounds which can cause liver damage, with prolonged use (Van Wyk *et al.*, 2002). No renal toxicity has been reported in previous literature, which is supported by findings in the current study.

5.2.1.3.6. *Pelargonium sidoides*

In both assays, the aqueous and organic extracts of *P. sidoides* were considered non-toxic after 24 and 48 hours of continuous exposure (Table 5.1).

In previous studies, *P. sidoides* has been considered safe for consumption (Teschke *et al.*, 2012). There were reports on possible hepatotoxicity caused by the use of *P. sidoides*, however, the *P. sidoides* causality was ruled out soon after (Teschke *et al.*, 2012). It has been reported that consumption of *P. sidoides* has no toxic effect on the liver or any other organs in animals. Furthermore, *P. sidoides* has an excellent safety profile in both adults and children (Afrigetics, 2013). Patients are advised, however, not to take *P. sidoides* if they have serious kidney or liver disease (Van Wyk, 2005). It has also been reported that *P. sidoides* extract exhibits significant effects on nasal epithelial cells (Neugebauer *et al.*, 2005).

5.2.1.3.7. *Sutherlandia frutescens*

The aqueous and organic extracts of *S. frutescens* were tested individually in the BSLA for toxic properties and the aqueous extract demonstrated a 0.00% mortality. The organic extract, however, demonstrated a mortality of $13.46 \pm 0.58\%$ and $82.69 \pm 4.51\%$ after 24 and 48 hours, respectively, with an LC_{50} value of 0.45 ± 0.05 mg/ml for the latter period. According to the classification of toxicity by Bussmann *et al.* (2011) for the BSLA, *S. frutescens* organic extract demonstrated moderate toxicity in the current study, since the LC_{50} value fell within the range of 250 – 499 μ g/ml (Section 2.8.1.3). In the MTT assay, the aqueous and organic extract of *S. frutescens* demonstrated no toxicity (Table 5.1). This lack of inhibitory effect was also observed *in vitro* on proximal and distal tubule epithelial cells, LLC-PK1 and

MDBK, where the IC₅₀ values were 15 mg/ml and 7 mg/ml, respectively. The mechanism by which the cells were affected by high concentrations of extract appear to be due to the increased oxidative stress, altered mitochondrial membrane integrity, and ability to promote apoptosis in renal tubule epithelia (Phulukdaree *et al.*, 2010).

The plant has been considered safe due to the long history of use in South Africa without reports of any toxicity (Teschke *et al.*, 2012) and there have been no reports on toxic effects affecting healthy adults (Fu *et al.*, 2008). The study by Seier *et al.* (2002), which was coordinated by Dr. Matsabisa (IKS/MRC) was the first toxicity study of *Sutherlandia*. The study involved testing leaf extracts of *Sutherlandia microphylla* on vervet monkeys for three months to observe the changes in parameters such as haematological, biochemistry, physiological variables and urine analysis. It was found that even at nine times the recommended daily dose (9.0 mg/kg) for an adult human, no toxic effects or adverse effects on the observed parameters had occurred. No toxic effects on the liver, kidney, muscles, lungs, bone or biochemical parameters were identified, thereby supporting the safety profile of the plant, which was not fully supported by the findings in this study and therefore effects could be species-dependent.

5.2.2. Toxicity analysis of some notable combinations

Eight plant: antimicrobial combinations were found to be notable in the antimicrobial studies, as interactions occurred between the traditional and conventional treatments of the same types of infections (Chapter 4). As such, these combinations were assessed for toxicity, using both the BSLA and the MTT assay, with the results obtained for both assays recorded in Table 5.2.

5.2.2.1. *Agathosma betulina* in combination with ciprofloxacin

In the current study, the essential oil, aqueous and organic extract of *A. betulina* were tested in combination with ciprofloxacin for toxicity. In the BSLA, none of these combinations demonstrated toxicity after 24 and 48 hours of exposure of the brine-shrimp to the samples. It is interesting to note, that the essential oil of *A. betulina*, when tested individually, demonstrated 100% mortality in the BSLA, however, when in combination with ciprofloxacin, no toxicity against the brine-shrimp was seen. In the MTT assay, none of the three combinations demonstrated toxicity toward the human kidney epithelial cells (Table 5.2).

Table 5.2. Mortality \pm S.D (%) and cell death (%) \pm S.D results (n = 6) for the notable plant: antimicrobial combinations.

	Sample or combination	BSLA study		MTT study
		Mortality \pm S.D (%) ^a		Cell death \pm S.D (%) ^b
		After 24 hrs:	After 48 hrs:	After 48 hrs:
Essential oils	<i>A. betulina</i> + ciprofloxacin	0.00	0.00	0.10 \pm 0.01
	<i>A. afra</i> + ciprofloxacin	2.13 \pm 0.58	2.13 \pm 0.58	0.10 \pm 0.01
Aqueous extract	<i>A. betulina</i> + ciprofloxacin	0.00	0.00	0.10 \pm 0.01
	<i>A. afra</i> + ciprofloxacin	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + ciprofloxacin	0.00	31.58 \pm 1.53	0.10 \pm 0.01
	<i>A. linearis</i> + erythromycin	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + gentamicin	0.00	4.35 \pm 0.58	0.10 \pm 0.01
	<i>A. linearis</i> + penicillin	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + tetracycline	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + amphotericin	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + nystatin	0.00	0.00	73.76 \pm 3.36
	<i>S. frutescens</i> + ciprofloxacin	0.00	0.00	0.10 \pm 0.01
Organic extract	<i>A. betulina</i> + ciprofloxacin	0.00	0.00	0.10 \pm 0.01
	<i>A. afra</i> + ciprofloxacin	0.00	1.25 \pm 1.00	0.10 \pm 0.01
	<i>A. linearis</i> + ciprofloxacin	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + erythromycin	0.00	6.67 \pm 0.58	0.10 \pm 0.01
	<i>A. linearis</i> + gentamicin	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + penicillin	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + tetracycline	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + amphotericin	0.00	0.00	0.10 \pm 0.01
	<i>A. linearis</i> + nystatin	0.00	0.00	56.88 \pm 6.61
	<i>S. frutescens</i> + ciprofloxacin	2.55 \pm 0.58	39.49 \pm 2.08	0.10 \pm 0.01

^a = Tested at a concentration of 1 mg/ml; ^b = Tested at a concentration of 100 μ g/ml; S.D = standard deviation.

5.2.2.2. *Artemisia afra* in combination with ciprofloxacin

In the current study, *A. afra* essential oil, aqueous and organic extract were tested for toxicity in combination with ciprofloxacin. In the BSLA, the essential oil combination demonstrated a 2.31 \pm 0.58% mortality of brine-shrimp within the first 24 hours of exposure to the

combination, after which, no further mortalities occurred. Both the aqueous and organic extract: ciprofloxacin combinations resulted in a percentage mortality well below 50%, indicating a favourable safety profile. In the MTT assay, no toxicity was seen for these three combinations of *A. afra* with ciprofloxacin towards the human kidney epithelial cells (Table 5.2).

5.2.2.3. *Aspalathus linearis* in combination with all seven antimicrobials

In the current study, *A. linearis* was tested in combination with all seven of the selected conventional antimicrobials, since it is such a commonly consumed plant and is not only consumed by users of traditional medicine, but the rest of the population in South Africa too.

In the BSLA, all combinations with the aqueous extract were found to have a 0.00% mortality against the brine-shrimp, except for the combinations of the aqueous extract with ciprofloxacin ($31.58 \pm 1.53\%$ mortality) and gentamicin ($4.35 \pm 0.58\%$ mortality), where the toxicity was only seen after 48 hours of exposure to the combinations. The organic extract combinations with the seven antimicrobials also showed a 0.00% mortality in the BSLA, except for the combination with erythromycin, where a $6.67 \pm 0.58\%$ mortality was seen after 48 hours. However, the degree of mortality observed for all three of these combinations does not warrant classifying the interactions as toxic and should not have had an influence on the antimicrobial inhibitory effect observed with these interactions. In the MTT assay, none of the combinations of the aqueous and organic extract of *A. linearis* in combination with the antimicrobials were found to be toxic against the human kidney epithelial cells, except for the combination with the antifungal, nystatin, where the aqueous and organic extract of *A. linearis* with nystatin demonstrated a cellular inhibition of $73.76 \pm 3.36\%$ and $56.88 \pm 6.61\%$ towards the human kidney epithelial cells, respectively (Table 5.2).

Nystatin entering the GIT is excreted unchanged, however, the possibility for other cellular interactions on the GIT should be considered. The possibility of rooibos facilitating increased uptake of nystatin through the GI wall into the systemic circulation should also be considered. An *in vitro* study by Tarirai *et al.* (2012) using Caco-2 cell monolayers and excised porcine jejunum tissue, has already shown that rooibos tea can have an effect on the intestinal absorptive profile of the conventional drug, cimetidine. Rooibos could also facilitate the transport of nystatin across membranes to increase the risk of toxicity. As such extended *in vivo* studies on the pharmacokinetic and pharmacodynamic properties of the combinations are warranted.

Since a cell inhibition greater than 50% was observed for the *A. linearis*: nystatin combinations, they were examined in more detail using a varied ratio study. From these studies, it was evident that the only ratio that was found to be toxic was the ratio consisting of equal volumes of each agent in the combination (50:50 μ l) ratio for both the aqueous and organic extract combination with nystatin, which corresponds to 0.05mg/ml of nystatin and 16.00 mg/ml of *A. linearis* extract. From the varied ratio study, it is also evident that the closer the ratios approach the 50:50 μ l ratio, the lower the cellular viability (Figure 5.1).

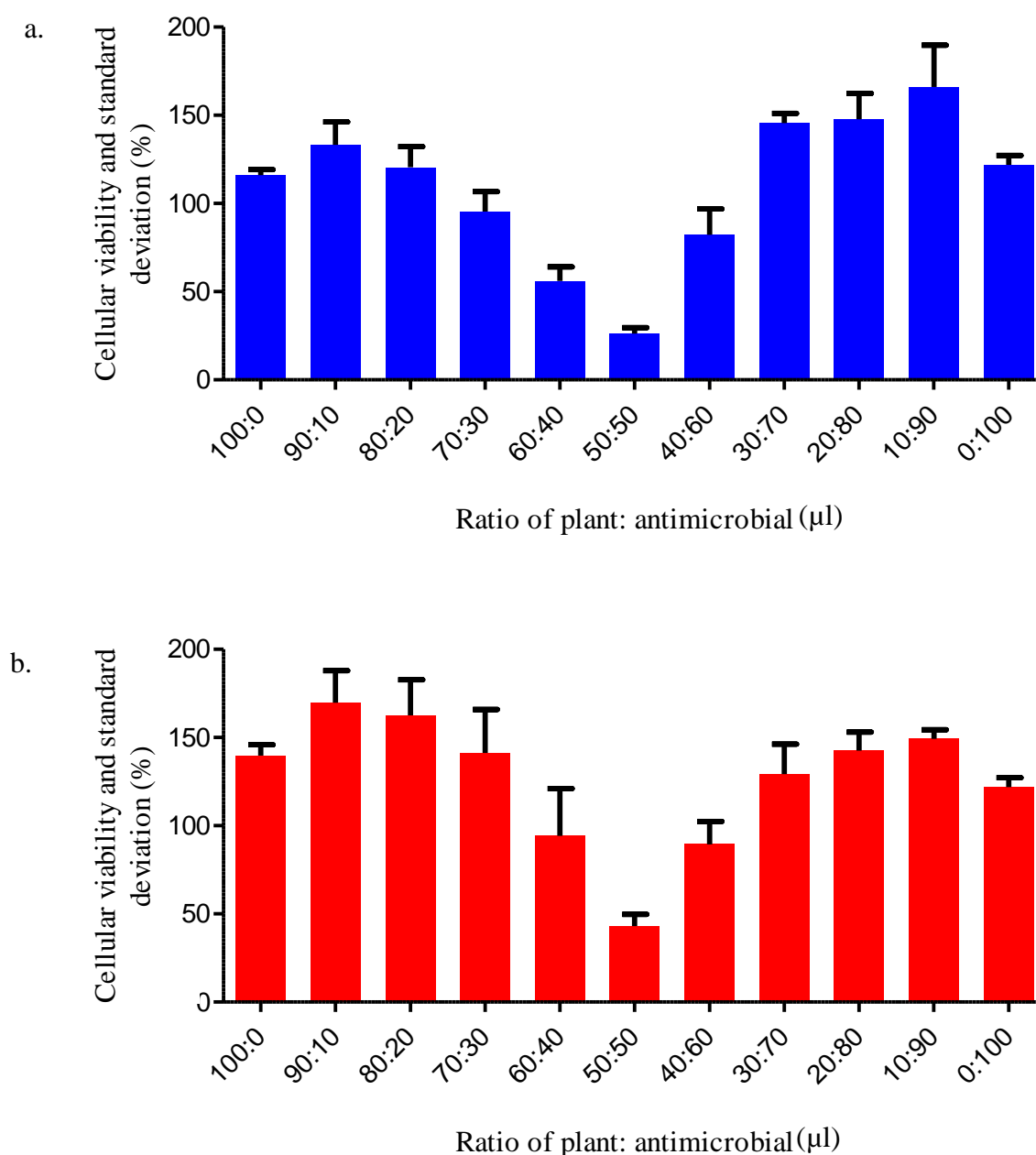


Figure 5.1. Varied ratios of *A. linearis* aqueous (a) and organic (b) extract: nystatin, and the corresponding cellular viability (n = 6).

In the antimicrobial combination studies (Chapter 4), the combination of *A. linearis* (aqueous and organic extract) with penicillin G demonstrated a mostly synergistic profile against the three tested pathogens (Table 4.10). In the toxicity screening of these two combinations, no toxic effect was seen in the BSLA or the MTT assay (Table 5.2), therefore the synergistic profile observed in the antimicrobial studies cannot be as a result of non-specific toxicity of the combination, but rather due to a more specific action against the microbe.

5.2.2.4 *Sutherlandia frutescens* in combination with ciprofloxacin

In the current study, the aqueous and organic extract of *S. frutescens* was tested for toxicity in combination with ciprofloxacin. In the BSLA, the aqueous extract combination demonstrated no toxicity, with a 0.00% mortality after 24 and 48 hours. However, the organic extract combination demonstrated a mortality of $2.55 \pm 0.58\%$ and $39.49 \pm 8.17\%$, after 24 and 48 hours, respectively. This combination was still not considered to be of significant toxicity, as the calculated mortality was below 50%. In the MTT assay, no toxicity was seen against the human kidney epithelial cells (Table 5.2).

5.3. Conclusions

- In the BSLA, the only toxicity seen for the medicinal plants was the essential oil of *A. betulina* (100% mortality, LC_{50} of 0.31 ± 0.03 mg/ml), the organic extract of *L. javanica* ($70.13 \pm 5.29\%$ mortality, LC_{50} of 0.51 ± 0.03 mg/ml) and the organic extract of *S. frutescens* ($82.69 \pm 4.51\%$ mortality, LC_{50} of 0.45 ± 0.05 mg/ml).
- In the whole-organism BSLA, no antimicrobials demonstrated toxicity, when tested at a concentration of 1.00 mg/ml and no plant: conventional antimicrobial combinations were found to be toxic either.
- In the cellular viability (MTT) assay, none of the plant and antimicrobial samples demonstrated toxicity when tested independently was found notable enough for IC_{50} determination.

- The only combination found to show toxicity in the MTT assay was between *A. linearis* (aqueous and organic extract) and nystatin, with cell inhibitions of $73.76 \pm 3.36\%$ and $56.88 \pm 6.61\%$ for the aqueous and organic extract combination with nystatin, respectively.
- In the varied ratio study of *A. linearis* (aqueous and organic extract) with nystatin, it was found that only the 50:50 μl ratio was found to be toxic. It was also noted that as the ratios approached the 50:50 μl ratio more closely, the cellular viabilities were reduced.

Chapter 6

Conclusion and future recommendations

6.1. Summary of dissertation

Many studies arising from the concern for interactions between orthodox drugs and natural products have provided evidence that interactions do most certainly occur between natural and conventional therapies, which is a major public health concern. Therefore, the aim of this study was to investigate the interactions between conventional antimicrobials and medicinal plants, in order to evaluate the effect that medicinal plants pose on the overall antimicrobial therapeutic effect of conventional antimicrobials. Combination therapy is a very popular line of investigation, since it provides a potential new avenue for the identification of more effective antimicrobials, in order to address antimicrobial resistance.

The current study varies from previous investigations, in that no combination studies have evaluated the effects of commercially relevant southern African medicinal plants on conventional antimicrobial therapies. The identification of interactions can be advantageous (synergistic) by providing new alternatives to combat resistance, but can also be harmful, by reducing the efficacy of current antimicrobials (antagonistic interactions), thereby contributing to an increase in resistance. Not only could there be an effect on efficacy, but possible toxicity could also arise.

Therefore, this study evaluated the potential for toxicity of the combinations that were found to be notable in the antimicrobial analysis. Figure 6.1 illustrates an overview of the study, where certain results have been highlighted. The objectives that were achieved in order to fulfil the aim of the study have also been discussed in more detail.

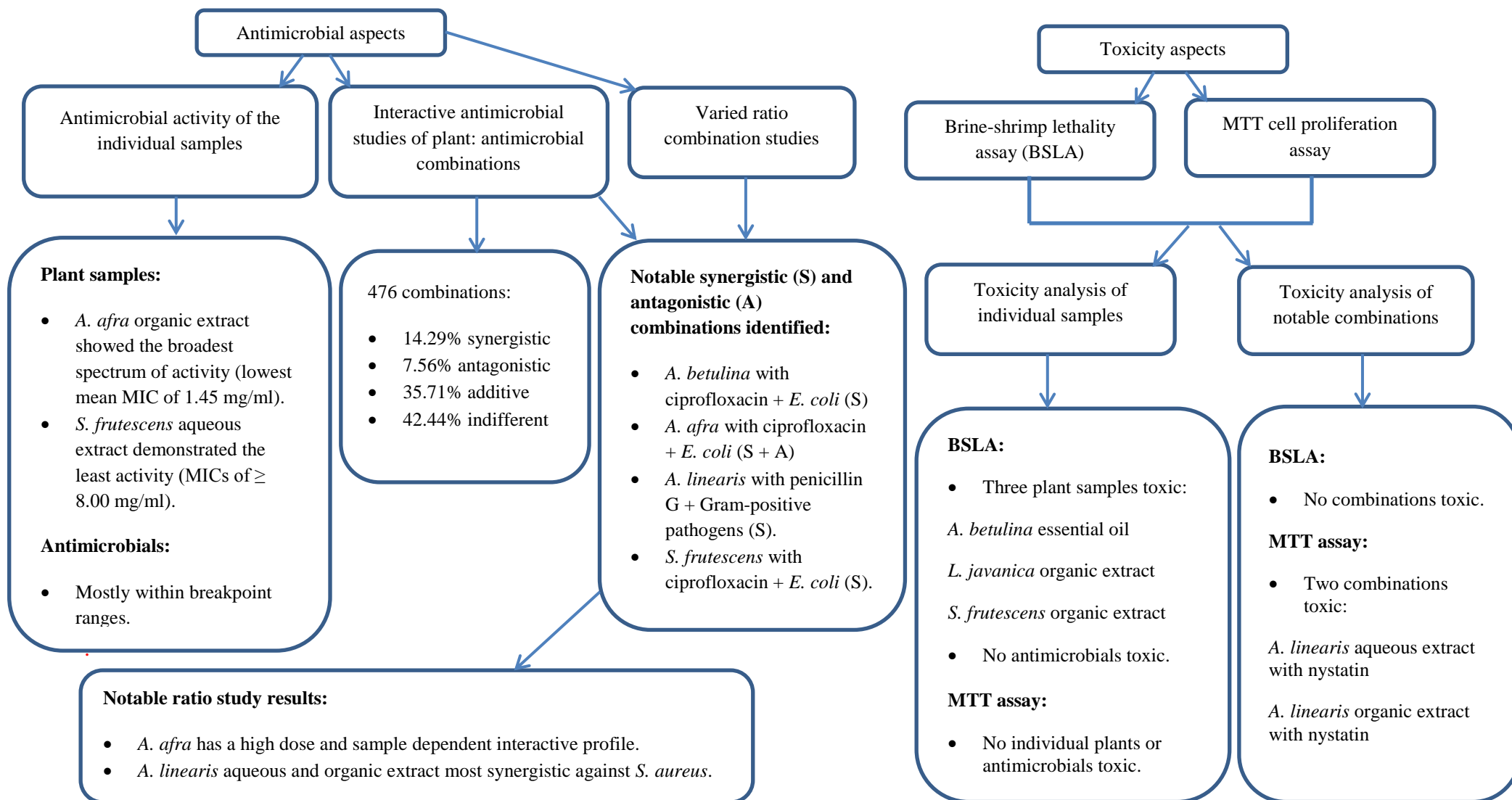


Figure 6.1. Outline of study with highlighted results for selected aspects.

6.1.1. Antimicrobial activity of the individual plant and antimicrobial samples

As expected, the antimicrobial activities of the conventional antimicrobials (Table 3.1) were found to be far superior to the medicinal plants (Table 3.3), when tested against a series of pathogens. Most of the medicinal plants demonstrated weak antimicrobial activity (Table 3.3) against the tested pathogens, which is in accordance with literature (Appendix F.1 – F.7).

The MIC results obtained for the conventional antimicrobials (Table 3.1) were compared with breakpoint expectation ranges (Table 3.2), to ensure that the antimicrobials were performing against the test micro-organisms as expected. All the antimicrobials were found to have MIC values within these breakpoint ranges, except for tetracycline, when tested against *E. faecalis* and *P. aeruginosa*, where these two strains were found to show an enhanced susceptibility towards tetracycline.

The aqueous and organic extracts of each medicinal plant, as well as the essential oil of the aromatic plants were tested for antimicrobial activity. The organic extract of *L. javanica* when tested against *S. aureus* (lowest MIC of 0.25 mg/ml), demonstrated the MIC value most noteworthy amongst all the medicinal plants when tested against the selected pathogens. The organic extract of *A. afra* was found to exhibit the broadest spectrum of antimicrobial activity, across the eight tested pathogens, with the lowest mean MIC of 1.45 mg/ml. The aqueous extract of *S. frutescens*, however, demonstrated the least antimicrobial activity, with a MIC value of ≥ 8.00 mg/ml against all eight test pathogens (Table 3.3).

6.1.2. Antimicrobial combination studies

All the plant samples were tested in combination with the conventional antimicrobials against the relevant pathogens, which resulted in the testing of a total of 476 combinations. Of these combinations, 14.29% were found to be synergistic, 7.56% were antagonistic, 35.71% were additive and 42.44% were found to be indifferent in nature (Figure 4.5). Therefore, the majority of the combinations tested were indifferent. Furthermore, many combinations were also additive in nature. This alleviates some of the concern related to the concurrent use of these agents, since no advantage or disadvantage is associated with these two types of interactions. However, a few notable synergistic and antagonistic interactions were identified, which could have an impact on conventional treatment regimens.

The combination of the conventional agent, ciprofloxacin, was found to be synergistic against the common causative micro-organism of UTI's, *E. coli*, in combination with two medicinal

plants (*A. betulina* and *S. frutescens*). The aqueous and organic extract of *A. betulina*, which are the traditional forms for consumption of the plant for UTI's, demonstrated a tentative synergistic interaction in combination with ciprofloxacin, when tested against *E. coli* (Table 4.2). The organic extract of *S. frutescens*, which is the traditional form for consumption for UTI's, demonstrated a favourable Σ FIC of 0.28 (synergistic interaction) when in combination with ciprofloxacin and tested against *E. coli* (Table 4.20).

Artemisia afra and ciprofloxacin are common traditional and conventional treatments for GI infections, respectively. These infections are very often caused by the consumption of food or water contaminated with *E. coli*. In this study, the combination of *A. afra* with ciprofloxacin demonstrated a varied interactive profile against *E. coli*, ranging from synergistic to antagonistic, depending on the dose, as well as the sample type (essential oil, aqueous or organic extract). *Artemisia afra* aqueous extract in combination with ciprofloxacin provided a notable antagonistic interaction (Σ FIC of 8.55) when tested against *E. coli*, strangely though, the organic extract and essential oil demonstrated a synergistic interaction (Σ FIC of 0.27 for both combinations) (Table 4.8). The combination with the essential oil is not as therapeutically relevant as that of the aqueous and organic extract combination, since the essential oil is not orally ingested for GI complaints.

The combination of *A. linearis* (rooibos) with penicillin G provided a mostly synergistic interactive profile against the three tested Gram-positive micro-organisms (*S. aureus*, *B. cereus* and *E. faecalis*), with Σ FIC values ranging from 0.01 (synergistic) to 0.94 (additive) (Table 4.10). In a varied ratio study, *A. linearis* combined with penicillin G was found to be mostly synergistic against *S. aureus*, with six of the nine ratios showing synergy for the aqueous extract: conventional antimicrobial combination and all nine of the ratios showing synergy for the organic extract: conventional antimicrobial combination (Figure 4.3). However, large quantities of rooibos would need to be consumed in order to provide the synergistic effect. For example, the ratio containing equal volumes of rooibos and penicillin would require approximately eight cups of rooibos to be consumed (Table 2.4).

As a healthcare professional, the results from this current study provide relief related to the concurrent use of traditional and conventional treatments for infections, however, the few notable interactions could impact on the efficacy of the conventional antimicrobials. This would need to be confirmed by *in vivo* testing, before these findings could be considered relevant in clinical practice.

6.1.3. Toxicity analysis of individual samples and notable combinations

In the whole-organism, brine-shrimp assay, none of the notable combinations that were tested demonstrated toxicity. However, some of the individual plant samples exhibited toxicity, namely *A. betulina* essential oil (100% mortality, LC₅₀ of 0.31 ± 0.03 mg/ml) and the organic extract of *L. javanica* (70.13% \pm 5.29% mortality, LC₅₀ of 0.51 ± 0.03 mg/ml) and *S. frutescens* (82.69% \pm 4.51% mortality, LC₅₀ of 0.45 ± 0.05 mg/ml) (Table 5.2).

In the cellular viability assay, the individual plant and antimicrobial samples demonstrated no toxic effects. When evaluating the toxicity of the notable combinations, only two combinations were found to be toxic, which was seen with *A. linearis* (aqueous and organic extract) in combination with nystatin, with a cell inhibition of $73.76 \pm 3.36\%$ and $56.88 \pm 6.61\%$, respectively (Table 5.2).

Therefore, the majority of the individual samples, as well as the notable plant: antimicrobial combinations were found to be non-toxic, except for the combination of *A. linearis* with nystatin, which would need further *in vivo* testing before the findings can be taken into account in clinical practice.

6.2. Future recommendations

The findings in this study are the first to provide *in vitro* scientific information pertaining to the combination of some of the South African traditional medicinal plants with conventional antimicrobials. There is still much research needed to further support these findings, as well as to contribute further to fill this void. Therefore, a few recommendations have been provided for future studies.

6.2.1. Mechanism of action studies

The concept of how plant extracts enhance the antimicrobial effect of available antibiotics and antifungals has not been explored in great depth (Adwan *et al.*, 2010), therefore, mechanism of actions studies are warranted. The mechanism of action of the various conventional antimicrobials have already been well investigated (Appendix G.1 – G.7). Cowan (1999) has provided a brief review of some of the most popular natural products that possess antimicrobial activity and their proposed mechanisms of action. The mechanism of action which allows for antimicrobial activity is often not as well defined for plants as it is for conventional antimicrobials. However, some studies have identified and isolated the chemical

compounds within the complex plant mixtures, which possess antimicrobial activity, for example phenols, terpenoids and alkaloids, where mechanisms of action have already been defined (Cowan, 1999). From the review by Cowan (1999), it is evident that the plant compounds exert antimicrobial activity in a very similar way to that of conventional antimicrobials. For example, via the disruption of the bacterial cell membrane or cell wall, inhibiting protein synthesis or even inactivating bacterial enzymes. The mechanism of action for antimicrobial activity of the individual conventional antimicrobials and medicinal plants are relatively well-known. However, the mechanism of action providing the interactions exhibited by the combinations needs to be investigated further, as it cannot be assumed that since the mechanism of action information is available for both the plants and antimicrobials, that one can postulate the outcome. If it is found in *in vivo* testing that plant and antimicrobials with similar mechanisms of actions (individually) provide a synergistic effect, it will then only allow for the postulation as to why the interactions are occurring and can aid in future herb-drug interaction interpretations. This may assist in preventing similar interactions from occurring. Synergistic interactions may also occur between agents which do not possess the same mechanism of action. A common example of this is seen with the conventional aminoglycosides and penicillins. The penicillin is responsible for perforating the bacterial cell wall, which then allows for a higher concentration of aminoglycoside to enter the bacterial cell, to target the intra-bacterial site of action, namely protein synthesis (SAMF, 2012).

In another study investigating combinations of conventional antimicrobials with natural products, it has been found that protein synthesis inhibitors, such as the aminoglycosides and macrolides, have strong synergistic interactions with plant extracts, whereas the nucleic acid synthesis inhibitors have been found to show very weak synergistic interactions with plant products (Betoni *et al.*, 2006). In this current study, the protein synthesis inhibitors (erythromycin, gentamicin and tetracycline) did show some synergy in combination with the plant samples; however, the cell wall synthesis inhibitor, penicillin G, demonstrated the highest synergy. Ciprofloxacin, which can be classified as a nucleic acid synthesis inhibitor due to its inhibition of DNA gyrase, did not follow the above assessment, since 16.67% of the interactions were found to be synergistic in nature. Ciprofloxacin, however, showed a higher synergy in comparison to some of the protein synthesis inhibitors. Therefore, this concept needs to be addressed with further mechanism of action studies for the combinations evaluated in this study.

6.2.2. Combinations of South African medicinal plants with other conventional drugs

From the results obtained in this current study, it is clear that medicinal plants can have an effect on conventional therapies, when used concurrently. It has already been found that some South African medicinal plants (*S. frutescens*, *A. linearis* and *H. hemerocallidea*) have interactions with other antimicrobials, such as antivirals, due to their ability to regulate hepatic CYP450 enzymes (Mills *et al.*, 2005; Matsuda *et al.*, 2007). Since plants have the ability to provide an array of effects on the human biological systems, it is necessary to not only focus on combinations with antimicrobials, but with other classes of conventional drugs too.

6.2.3. *In vivo* studies

Since this study focused on the *in vitro* activity for plant: conventional antimicrobial combinations, the interactive profile cannot be assumed to be the same when within the human body. Therefore, further *in vivo* studies are necessary, to confirm the *in vitro* interactive profiles of the combinations. Since plants have such a complex composition, they are able to have numerous biological effects, allowing for a holistic approach to treatment (Van Vuuren, 2007). These concomitant effects are not all portrayed within the *in vitro* model of testing and therefore further animal and human studies are necessary to account for all the modes of action which a plant mixture can have in the human body (Van Vuuren, 2007). This study has, however, provided a good starting point using preliminary *in vitro* screening, as a means to determine which combinations may be advantageous or not recommended in combination. Also, *in vivo* animal studies would not be feasible in the testing of all 476 combinations. Whereas the *in vitro* screening approach, as studied here, provides an excellent overview of the different interactive profiles, and provides a starting point for further follow-up *in vivo* studies.

6.2.4. Formulation studies

The final step to ensure a comprehensive approach to the concept of medicinal plant: conventional antimicrobial combinations would be to include formulation studies, since synergistic combinations could assist in overcoming the problem of antimicrobial resistance. Therefore, it would be ideal to be able to create a formulation, preferably in tablet form, containing both plant and conventional antimicrobial. The problems that could arise in combining these two forms of medicine include stability and standardisation issues.

However, the benefits of such combinations include a more natural approach to antimicrobial therapies and could also result in less toxicity and side effects due to dose reductions.

6.2.5. Healthcare professional and public awareness

Healthcare professionals are mostly only aware of a few natural products that can affect conventional therapies, such as St. John's wort and grapefruit juice. However, very little is known on the interactions between traditional medicinal plants and conventional therapies, which is worrisome, since many South Africans use these two forms of healthcare concurrently. Therefore, the undergraduate programmes for medical and pharmacy students should ideally contain some coursework on interactions that may occur with traditional and conventional medicine combinations, and not only focus on the interactive profiles of common herbal preparations with conventional drugs. The public also needs to be made aware of the possibility of interactions between natural products and conventional drugs, since very often, the assumption is that natural products are safe and free from harm. Due to this assumption, very few patients think it necessary to mention natural product or traditional medicine use when consulting with a healthcare provider (Ioannides, 2002), which further increases the possibility for interactions to occur. Therefore, to overcome this problem, it should always be recommended that when recording patient information, that natural product or traditional medicinal use is queried and recorded. This should be as important in the history taking process as when asking the patient if they use any other medication or if they have any allergies.

6.2.6. Regulations regarding traditional medicine

Regulations in most countries have not placed emphasis on the scientific demonstration of efficacy, safety and quality of natural products due to the way in which these natural products are promoted as safe and harmless (Homsy *et al.*, 2004). Government legislation and regulations regarding natural product and traditional remedies are necessary, in order to avoid future adverse interactions. These regulations may include more comprehensive labelling of natural product remedies to include warnings of interactions with conventional drugs; or informing and educating traditional healers on these interactions, so that the information can be relayed to the patients (Ioannides, 2002).

6.3. Conclusions

The majority of the plant: antimicrobial combinations tested in the current study demonstrated indifferent interactions, which alleviates concern related to concurrent use. However, a few notable synergistic and antagonistic interactions were also identified, which could have a considerable impact on treatment regimens related to infection control. The possibility for interactions between conventional and traditional or herbal medicines has not only been emphasized in this study, but in others too (Ioannides, 2002; Fasinu *et al.*, 2012). Therefore, there is definitely a need for more detailed research this field, since there is insufficient information available for clinical applications. It is also necessary for findings to be relayed to healthcare professionals and the public (Fasinu *et al.*, 2012).

Further scientific research directed at traditional medicine can also allow for recognition of the efficacy of this type of medicine, which could further result in the acceptance of medicinal plants into orthodox medicine (Van Vuuren, 2007). Combination studies have also resulted in the identification of new alternatives to current conventional antimicrobials, to which resistance has developed. This concept has extended to natural products and medicinal plants in combination with conventional therapies, which supports the claim by Tyler (1999), that phytomedicines should be integrated into orthodox medicine, where these agents can be prescribed or recommended by a healthcare practitioner, to be used solely on its own, or in combination with conventional drugs. Some of the plants investigated in this study have certainly shown their ability in potentiating the antimicrobial activity of conventional antibiotics and antifungals, and this is the first step in future endeavours within this field of research.

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Appendix A

Abstract for oral presentation at APSSA conference

Antimicrobial efficacy of conventional antimicrobial agents in combination with commercially relevant southern African medicinal plants.

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Purpose:

Antimicrobial agents are one of the most commonly prescribed drugs. Furthermore, in South Africa, at least 60% of the population consult traditional healers. There is thus a considerable possibility for the concomitant use of antimicrobial agents and traditional medicine. The purpose of this study was to evaluate the interactive antimicrobial profiles against a range of pathogens, when seven antimicrobial agents were combined with the essential oils, aqueous and organic extracts of seven plants, classified as the most commonly used and traded in South Africa.

Methods:

The antimicrobial activity of the plant samples and conventional antimicrobials were evaluated, alone and in combination, using the minimum inhibitory concentration (MIC) assay against two yeasts, three Gram positive and three Gram negative bacteria. The combinations were further assessed using the fractional inhibitory concentration (Σ FIC) calculation. Combinations demonstrating significant synergistic or antagonistic interactions were further evaluated in varying concentrations, for isobologram construction.

Results:

Additive (25.0%) and non-interactive (50.8%) effects were observed, but most importantly, synergistic (14.3%) and antagonistic (9.8%) interactions were also noted when the antimicrobials were combined with the medicinal plants. For all combinations containing penicillin, synergistic interactions were mostly noted (38.8%). However, combinations

containing gentamicin, showed the most antagonism (25.9%). For therapeutic purposes, a few significant interactions were identified. Ciprofloxacin, commonly used for urinary tract infections (UTI's) demonstrated synergy against *Escherichia coli*, when in combination with *Agathosma betulina* as well as *Sutherlandia frutescens* (Σ FIC 0.26 and 0.28 respectively), where both plants are commonly used in the treatment of UTI's. Conversely, tetracycline, which is commonly used for skin infections, demonstrated antagonism against *Pseudomonas aeruginosa*, when in combination with *A. betulina* and *Aloe ferox* (Σ FIC 8.81 and 8.84 respectively), which are plants often used to treat skin conditions.

Conclusion:

No specific pattern (synergistic or antagonistic) exists when the two forms of healthcare are used concurrently. However, a few significant interactions were identified, which could have a noteworthy impact on conventional treatment regimens and thus further *in vivo* investigations and toxicity profiling for these combinations are warranted.

Appendix B

Abstract for oral presentation at the School of Therapeutic Sciences (STS) 2013 research day

What are the implications of combining conventional antimicrobials with traditional medicinal plants?

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Purpose:

In South Africa, at least 60% of the population consult traditional healers for their primary healthcare needs, where very often, medicinal plants are provided as a treatment option. It has been acknowledged that in some of the finest hospitals in South Africa, concurrent use of conventional and traditional medicine still occurs. There is a considerable possibility for the concurrent use of antimicrobial agents and traditional medicine. The purpose of this study was to evaluate the interactive antimicrobial and toxicity profiles against a range of pathogens, when seven conventional antimicrobial agents (amphotericin B, ciprofloxacin, erythromycin, gentamicin, nystatin, penicillin G and tetracycline) were combined with the essential oils, aqueous and organic extracts of seven medicinal plants (*Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Aspalathus linearis*, *Lippia javanica*, *Pelargonium sidoides* and *Sutherlandia frutescens*).

Methods:

The antimicrobial activity of the plant samples and conventional antimicrobials were evaluated, alone and in combination, using the minimum inhibitory concentration (MIC) assay against two yeasts, three Gram-positive and three Gram-negative bacteria. The combinations were further evaluated using the fractional inhibitory concentration (Σ FIC) assessment. Combinations demonstrating notable synergistic or antagonistic interactions were

further evaluated in various ratios (isobolograms). Toxicity of the antimicrobials and plant samples were assessed individually and in combination using the brine-shrimp lethality assay (BSLA) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay on human kidney epithelial cells (Graham or HEK-293 cell line).

Results:

A total of 476 combinations were assessed for interactive antimicrobial potential. Of these combinations, 14.29% were synergistic, 7.56% were antagonistic, 35.71% were additive and 42.44% were indifferent in nature. The most notable interactions were that of *A. linearis* (aqueous and organic extract) with penicillin G, where synergy was most often seen against the Gram-positive micro-organisms, *Staphylococcus aureus* (Σ FIC of 0.01 for the organic extract and a tentative synergistic interpretation for the aqueous extract), *Bacillus cereus* (Σ FIC of 0.08 for the organic extract and a tentative synergistic interpretation for the aqueous extract) and *Enterococcus faecalis* (Σ FIC of 0.94 (additive) for the organic extract and 0.46 for the aqueous extract). In the BSLA, the notable interactions that were tested demonstrated no toxic effect. In the MTT cell proliferation assay, the only combination demonstrating a possible toxic effect was that of *A. linearis* (aqueous and organic extract) in combination with nystatin (cell inhibition of 73.76% and 56.88% respectively).

Conclusion:

Most interactions were found to be indifferent (42.44%), providing some relief for concerned concurrent users. However, synergistic and antagonistic interactions were also identified, which could have a considerable impact on treatment regimens. Antagonistic interactions could reduce the efficacy of the conventional antimicrobials. Synergistic interactions could enhance the effect of the conventional antimicrobials, and so, possible dose reductions would be necessary. The concurrent use of rooibos tea and conventional antimicrobials should be cautioned. The only combination found to be toxic, in the MTT assay, was that of *A. linearis* (aqueous and organic extract) in combination with nystatin.

Appendix C

Abstract for oral presentation at the Postgraduate Symposium at the University of Johannesburg (UJ)

Can traditional medicinal plants have an effect on conventional antimicrobial therapies?

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The use of medicinal plants as a source of healthcare plays a significant role in the cultural heritage of many South Africans, with at least 60% of the population consulting traditional healers. It has been acknowledged that in some of the finest hospitals in South Africa, concurrent use of conventional and traditional medicine still occurs. In orthodox medicine, antimicrobials such as antibiotics and antifungals, are amongst the most commonly prescribed groups of drugs. Therefore, there is a considerable possibility for the concurrent use of these two forms of healthcare. The aim of this study was to evaluate the interactive antimicrobial profiles, against a range of pathogens, as well as the toxicity profiles, when seven conventional antimicrobial agents (amphotericin B, ciprofloxacin, erythromycin, gentamicin, nystatin, penicillin G and tetracycline) were combined with the essential oils, aqueous and organic extracts of seven medicinal plants (*Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Aspalathus linearis*, *Lippia javanica*, *Pelargonium sidoides* and *Sutherlandia frutescens*). The antimicrobial activity of the plant samples and conventional antimicrobials were evaluated, alone and in combination, using the minimum inhibitory concentration (MIC) assay against two yeasts, three Gram-positive and three Gram-negative bacteria. The combinations were further evaluated using the fractional inhibitory concentration (Σ FIC)

assessment. Combinations demonstrating notable synergistic or antagonistic interactions were further evaluated in various ratios (isobolograms). Toxicity of the antimicrobials and plant samples were assessed, individually and in combination, using the brine-shrimp lethality assay (BSLA) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay on human kidney epithelial cells (Graham or HEK-293 cell line). A total of 476 combinations were assessed for interactive antimicrobial potential. Of these combinations, 14.29% were synergistic, 7.56% antagonistic, 35.71% additive and 42.44% indifferent in nature. The most notable interactions were that of *A. linearis* (aqueous and organic extract) with penicillin G, where a synergistic profile was most often seen against the three tested Gram-positive micro-organisms (*Staphylococcus aureus*, *Bacillus cereus* and *Enterococcus faecalis*), with Σ FIC values ranging from 0.01 to 0.94 (additive). The synergistic and antagonistic interactions identified in this study could have a considerable impact on conventional treatment regimens. Antagonistic interactions could reduce the efficacy of the conventional antimicrobials. Synergistic interactions could enhance the effect of the conventional antimicrobials, and so, possible dose reductions would be necessary. In the BSLA, the notable interactions that were tested demonstrated no toxic effect. In the MTT cell proliferation assay, the only combination demonstrating possible toxicity was that of *A. linearis* (aqueous and organic extract) in combination with nystatin (cell inhibition of 73.76% and 56.88% respectively), therefore concurrent use should be cautioned.

Appendix D

Abstract for publication submitted to the *South African Journal of Botany*

Can rooibos (*Aspalathus linearis*) tea have an effect on conventional antimicrobial therapies?

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ABSTRACT

One of the most commonly consumed and commercially relevant herbal teas in South Africa is rooibos tea, prepared from the plant, *Aspalathus linearis* (Burm. F.) Dahlg. In orthodox medicine, antimicrobial agents are amongst the most commonly prescribed groups of drugs and thus there is a considerable possibility for the concurrent use of these drugs with the highly popular beverage, rooibos tea. Therefore, the aim of this study was to investigate the interactive antimicrobial and toxicity profiles of *A. linearis* (aqueous and organic extract), when combined with seven conventional antimicrobials (ciprofloxacin, erythromycin, gentamicin, penicillin G, tetracycline, amphotericin B and nystatin). The antimicrobial activity of *A. linearis* was evaluated, independently and in combination, using the minimum inhibitory concentration (MIC) assay against two yeasts, three Gram-positive and three Gram-negative bacteria. The interactions were further evaluated using the sum of the fractional inhibitory concentration (Σ FIC) assessment. Combinations demonstrating notable synergistic or antagonistic interactions were investigated in various ratios (isobolograms). The toxicity of *A. linearis* extracts and antimicrobials, were assessed independently and in combination, using the brine shrimp lethality assay (BSLA), and the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay on the human HEK-293 cell line. *Aspalathus linearis* (aqueous and organic extract) with penicillin G demonstrated the most notable interactions, when tested against the Gram-positive bacteria,

with Σ FIC values ranging from 0.01 (synergistic) to 0.94 (additive). Varied ratio studies of this combination were most synergistic against *Staphylococcus aureus*. Four antagonistic combinations were identified, and were found against the Gram-negative bacteria and yeasts. In the BSLA, no toxic combinations were identified. However, in the MTT assay, two combinations were found to demonstrate a possible toxic effect (*A. linearis* aqueous and organic extract with nystatin), with inhibitory effects of $73.76 \pm 3.36\%$ and $56.88 \pm 6.61\%$, respectively, thus warranting further *in vivo* studies.

Appendix E

Abstract for publication submitted to the *South African Journal of Botany*

Interactive antimicrobial and toxicity profiles of conventional antimicrobials with southern African medicinal plants.

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ABSTRACT

Medicinal plant use plays an important role in the healthcare of many South Africans. Furthermore, in orthodox medicine, conventional antimicrobial agents are amongst the most commonly prescribed groups of drugs. Therefore, due to the prevalence of use of these two forms of healthcare, there is a high probability for their concurrent use. Thus, the aim of this study was to evaluate the interactive antimicrobial and toxicity profiles of six southern African medicinal plants (*Agathosma betulina*, *Aloe ferox*, *Artemisia afra*, *Lippia javanica*, *Pelargonium sidoides* and *Sutherlandia frutescens*) when combined with seven conventional antimicrobials (ciprofloxacin, erythromycin, gentamicin, penicillin G, tetracycline, amphotericin B and nystatin). Antimicrobial activity was assessed using the minimum inhibitory concentration (MIC) assay against a range of pathogens and interactions were further classified using the sum of the fractional inhibitory concentration (Σ FIC). Notable synergistic or antagonistic interactions were studied at various ratios (isobolograms). The toxicity of the individual samples, as well as the notable combinations, were assessed using the brine-shrimp lethality assay (BSLA) and the 3-(4,5 dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) assay on the HEK-293 human cell line. Of the 420 antimicrobial: plant combinations studied, 14.29% showed synergistic interactions, 7.56% antagonistic, 35.71% additive and 42.44% indifferent interactions. Some notable synergistic interactions (ciprofloxacin with *Agathosma betulina* and *Sutherlandia frutescens* against *Escherichia coli*) and antagonistic interactions (ciprofloxacin with *Artemisia afra* organic extract against *Escherichia coli*) were identified. None of the notable combinations were found to show toxicity in the BSLA or MTT assays. In conclusion, the majority of combinations were found to have no notable interaction, alleviating some concern related to the concurrent use of these two forms of healthcare.

Appendix F

Medicinal plants investigated in this study

Appendix F.1.

Agathosma betulina (Berg.) Pillans

**Family name**

Rutaceae

Vernacular names

Round leaf buchu (English)

boegoe, bergboegoe (Afrikaans)

ibuchu (Xhosa)

buchu (Khoi)

Figure F.1.1. *Agathosma betulina* [Photograph by Prof. A.M. Viljoen from Tshwane University of Technology (TUT)].

Botanical description

It is a shrub that grows approximately two metres in height. The leaves are usually broad, with a rounded apex. Flowers are small, white and star-shaped (Van Wyk *et al.*, 2009).

Geographical distribution

The plant occurs naturally in the mountains of the Western Cape (Van Wyk *et al.*, 2009).

Traditional medicinal use

Health tonic, diuretic, urinary tract infections (UTI's), haematuria, prostatitis, colds and flu, coughs, gout, fever, rheumatism, wounds, boils, rashes, bruises, burns, gastrointestinal (GI)

complaints, cholera, and the antibiotic protection of corpses (Pillans, 1950; Watt and Breyer-Brandwijk, 1962; Simpson, 1998, Van Wyk *et al.*, 2009; Van Wyk, 2011). The San also used the aromatic plant mixed with fat as a skin lubricant in the desert. Topically, the plant acted as an antibacterial, antifungal, insect repellent and deodorant (Van Wyk *et al.*, 1997).

Plant parts used

Dried leaves (Van Wyk *et al.*, 2009).

Dosage form and route of administration

It is usually administered orally as an aqueous infusion prepared from the leaves, which can sometimes be sweetened with the addition of brown sugar (Scott and Springfield, 2004a). Decoctions or alcoholic tinctures prepared in brandy (“boegoebrandewyn”) are also ingested orally. Buchu vinegar (“boegoe-asyn”), prepared from the leaves of the plant, is used topically for cleaning wounds (Watt and Breyer-Brandwijk, 1962; Van Wyk *et al.*, 2009). Products available on the market include buchu tea leaves and essential oil from Skimmelberg, as well as a kidney tonic and pancreas spray from Medico Herbs, Western Cape (Figure F.1.2).



Figure F.1.2. Commercial products containing *A. betulina*. A = Buchu tea leaves (Skimmelberg, 2011a); B = Buchu oil (Skimmelberg, 2011a); C = Buchu kidney tonic (Medico Herbs, 2009a); D = Buchu pancreas spray (Medico Herbs, 2009b).

Adverse reactions or toxic effects

Consumption of the plant has been associated with allergic reactions, which is due to the high eugenol content of the plant (Murakami *et al.*, 2005). GI disturbances if administered orally have also been reported. Prolonged use is not advised. Use in pregnancy and lactation is

contraindicated (Scott and Springfield, 2004a). No cytotoxicity was seen when the aqueous extract of *A. betulina* was tested on HeLa cells, derived from cervical cancer cells (May and Willuhn, 1978). The plant has demonstrated no toxic effect on human kidney cells ($IC_{50} > 100 \mu\text{g/ml}$) (Moolla, 2005).

Antimicrobial activity

Agathosma betulina has been described as having a general antimicrobial activity, which extends over a variety of micro-organisms. The antimicrobial efficacy has been accredited to the terpenoid content of the plant (Cowan, 1999). *Agathosma betulina* has shown no activity toward the Gram-negative bacterium, *Pseudomonas aeruginosa*. Low activity has been documented against other Gram-negative pathogens, such as *Escherichia coli* and *Klebsiella pneumoniae*, as well as against three Gram-positive pathogens, namely *Bacillus cereus*, *Enterococcus faecalis* and *Staphylococcus aureus* and the yeast, *Cryptococcus neoformans* (Lis-Balchin *et al.*, 2001; Moolla, 2005; Moolla and Viljoen, 2008).

History and commercialisation of the plant

Agathosma betulina was first used by the San and Khoi people and is an important plant in their cultural heritage. It was wild-harvested from 1820 and cultivated since 1970 (Van Wyk, 2011). The plant only became a well-known Cape medicine once the medicinal properties of the plant were discovered by the Dutch colonists. It has been considered as one of South Africa's most popular medicinal plants and is still widely used by many South Africans (Van Wyk *et al.*, 2009). Buchu was first introduced into Britain in 1790 and was soon considered an official remedy, where it was even published in British Pharmacopoeia (BP) (Scott and Springfield, 2004a). In Europe, there are numerous patented remedies containing buchu, such as teas and drops, which are available without a prescription for self-medication. It is now commonly available worldwide as a commercial product, in various forms such as a concentrated buchu infusion, liquid extract or buchu tincture, buchu oil, dried buchu leaves, buchu water and powdered buchu, all used for either the medicinal value or as a cosmetic or flavouring agent (Van Wyk *et al.*, 1997). In South Africa, the British Pharmaceutical Codex (BPC) quality buchu is found in many pharmacies. The unstandardised leaves of *A. betulina* can also sometimes be found in supermarkets (Scott and Springfield, 2004a).

Appendix F.2.

Aloe ferox Mill.



Family name

Asphodelaceae

Vernacular names

bitter aloe (English)

bitteraalwyn, Kaapse alwyn (Afrikaans)

umhlaba (Xhosa, Zulu, Sotho)

Figure F.2.1. *Aloe ferox* [Photograph by Prof. A.M. Viljoen (TUT)].

Botanical description

The plant has broad, fleshy leaves, usually pale green in colour, with a red tinge sometimes. Flowers are red or orange in colour and are found vertically erect from the base of leaves (Van Wyk *et al.*, 2009).

Geographical distribution

The plant is located along the eastern parts of South Africa (Van Wyk *et al.*, 2009).

Traditional medicinal use

Sexually transmitted infections (STI's), wounds, burns, sinusitis, conjunctivitis, constipation, hypertension, stress. The leaf is used for skin and hair treatments (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996; Van Wyk *et al.*, 2009; Van Wyk, 2011). The leaves are cut and applied directly to burns, insect bites, sores and sunburn. The plant has also been used for arthritis, toothache and stomach complaints (Crouch *et al.*, 2006). Leaf and stem decoctions are used as an emetic and the leaves and roots boiled in water are used for hypertension and stress (Pujol, 1990).

Plant parts used

Leaves, roots, juice or gel (Van Wyk *et al.*, 2009).

Dosage form and route of administration

Fresh juice from leaves or decoctions and powders from leaves or roots can be applied topically or sniffed (Hutchings *et al.*, 1996). Fresh bitter sap is also often instilled directly into the eye or nose (Van Wyk *et al.*, 2009). Products available on the market include a heel balm and a regenerating gel from Nature's Health Products South Africa and a health drink from Docsemur (Figure F.2.2).



Figure F.2.2. Commercial products containing *A. ferox*. A = *A. ferox* heel balm (Nature's Health, 2012a); B = *Aloe* regenerating gel (Nature's Health, 2012b); C = *Aloe* health drink (Docsemur, 2012).

Adverse reactions or toxic effects

There is no evidence of cytotoxicity when the plant is consumed in low doses (Kambizi and Afolayan, 2008; Wintola *et al.*, 2011). However, in overdose, toxicity occurs, demonstrated by similar effects seen in curare poisoning, which include joint weakness and partial paralysis. Overdose can also lead to nephritis and gastritis (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996). *Aloe ferox* has been considered generally safe, except for possible hypersensitivity, and should not be used during pregnancy (Van Wyk *et al.*, 2009). Aqueous extracts of *A. ferox* administered to constipated rats did not enlarge the liver or elevate liver enzymes and had no effect on haematological parameters (Wintola *et al.*, 2011). At 500 mg/kg, there was a lack of bacterial and mammalian cell genotoxicity (Andersen, 2007).

Antimicrobial activity

Aloe ferox has been found to be active against *Candida albicans*, *Neisseria gonorrhoea* and the *Herpes simplex* virus (Kambizi *et al.*, 2007; Van Wyk, 2011). *Aloe ferox* aqueous extract demonstrated no activity toward against *C. albicans*, whereas the methanol extract exhibited low activity at a concentration of 20 mg/ml. One of the major compounds in *A. ferox* is aloin, which was found to be poorly active against *C. albicans* at a concentration of 5 mg/ml (Kambizi and Afolayan, 2008).

History and commercialisation of the plant

The use of *A. ferox* for medicinal purposes has been illustrated in San rock paintings (Reynolds, 1950). However, plantations for commercial production of *A. ferox* only began in 1976. It is still a very important, widely used commercial medicine used in South Africa for the treatment of constipation (Van Wyk *et al.*, 2009). There is a large international market for *A. ferox* remedies. Exporting to Europe began as early as 1761. Locally, the remedies are marketed as *Aloe* lump or a tincture prepared from the *Aloe* lump (Van Wyk, 2011) and is most commonly sold on the South African market as a laxative by Lewenssens and Lennon (Van Wyk *et al.*, 2009). Many dermatological products containing *A. ferox* are available on the market, in the form of creams or cosmetics. Today, in South Africa, it is one of South Africa's main wild-harvested commercially traded species. The increase in demand for the plant has also resulted in increased economic gain for people in the rural areas, where the plant is most often harvested by these people (Chen *et al.*, 2012).

Appendix F.3.

Artemisia afra Jacq. ex Willd.



Family name

Asteraceae

Vernacular names

African wormwood (English)

wilde als (Afrikaans)

unhlonyane (Xhosa, Zulu)

lengana (Sotho)

Figure F.3.1. *Artemisia afra* [Photograph by Prof. S.F. Van Vuuren (WITS)].

Botanical description

It is a shrub growing up to two metres in height. The leaves are finely divided, providing a feathery appearance and are greenish-grey in colour. Flowers occur at the ends of the branches and are pale yellow (Van Wyk *et al.*, 2009).

Geographical distribution

The plant is found throughout all areas of South Africa and can even extend up northward towards Ethiopia (Van Wyk *et al.*, 2009).

Traditional medicinal use

Respiratory infections (coughs, colds, pneumonia, croup, whooping cough), asthma, hay fever, GI disorders, colic, heartburn, flatulence, gout, influenza, fever, diabetes, malaria, intestinal worms, haemorrhoids, headaches, bladder and kidney disorders, convulsions, loss of appetite, heart inflammation, rheumatism, stings and bites, wounds, mouth ulcers, gum problems, earache, blood purifier for the treatment of acne, boils and measles (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996; Felhaber, 1997; Thring and Weitz, 2006; Van Wyk *et al.*, 2009; Van Wyk, 2011).

Plant parts used

Leaves and sometimes roots (Van Wyk *et al.*, 2009).

Dosage form and route of administration

Aqueous infusions prepared from the leaves or decoctions prepared from the roots are orally administered. Aqueous preparations are also used as enemas (Dube, 2006). The bitter taste can be masked with the addition of sugar or honey. A poultice of leaves is often used for topical application (Watt and Breyer-Brandwijk, 1962). Vapour released from boiled leaves can also be inhaled (Van Wyk *et al.*, 2009). Products available on the market include tea leaves from Nature's Health Products South Africa and *A. afra* drops from Medico Herbs, Western Cape (Figure F.3.2).



Figure F.3.2. Commercial products containing *A. afra*. A = *A. afra* tea leaves (Nature's Health, 2012c); B = *A. afra* drops (Medico Herbs, 2009c).

Adverse reactions or toxic effects

The essential oil has been said to cause haemorrhagic nephritis, degenerative liver changes, pulmonary oedema and sometimes abortions in rabbits (Watt and Breyer-Brandwijk, 1962). Hallucinogenic effects have also been reported (Van Wyk *et al.*, 2002). Adverse effects and toxicity are mainly due to thujone poisoning when excessive amounts are consumed or if the plant is used for a prolonged period of time. Effects of thujone poisoning include vomiting, vertigo, convulsions, restlessness and hepatotoxicity (Van Wyk *et al.*, 2009). Aqueous extracts of *A. afra* have been found to be cytotoxic at high concentrations when tested against various cancer cells lines, such as cervical cancer cells (HeLa) and T-lymphoblastic

leukaemia cells (Jurkat E6-1, AA-2 and CEM-SS) (Treurnicht, 1997). Mativandlela *et al.* (2008) found cytotoxicity of the ethanolic extract of *A. afra* in Vero cells (kidney epithelial cells extracted from a monkey) (IC₅₀ value of 113.0 ± 2.05 µg/ml). It has been stated that the solubility of the toxic compound, thujone, in water is, however, very low and so the possibility of poisoning due to an aqueous extract is unlikely at recommended dosages (McGaw *et al.*, 2000). Mukinda and Syce (2007) studied the acute toxic effects of an aqueous extract of *A. afra* in rats, and observed a dose-dependent increase in mortalities. When evaluating the toxicity with chronic oral administration of low concentrations, no deaths occurred after three months and the rats appeared healthy. However, once concentrations started to exceed 1000 mg/kg, minor symptoms of toxicity were observed, such as diarrhoea. There was no effect seen on the kidney and liver function of the rats and it was even proposed that *A. afra* aqueous extract could have a hepatoprotective effect (Mukinda and Syce, 2007).

Antimicrobial activity

Antimicrobial activity extends over a range of bacteria and fungi (Graven *et al.*, 1992). Ethanolic extracts of *A. afra* were found to be active against *S. aureus* and *Bacillus subtilis*, but not against *E. coli* and *K. pneumoniae*. Aqueous and hexane extracts were not found to be active against any of the micro-organisms studied (McGaw *et al.*, 2000). *Artemisia afra* has also shown activity against *P. aeruginosa* and *C. albicans*. Weak activity has been documented against *C. neoformans* and *E. faecalis*. MIC values have ranged from 4.5 – 32 mg/ml against *S. aureus*, *Staphylococcus epidermidis*, *B. cereus*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *K. pneumoniae*, *C. albicans* and *C. neoformans* (Huffman *et al.*, 2002; Van Vuuren and Viljoen, 2006; Suliman *et al.*, 2010).

History and commercialisation of the plant

Artemisia afra is one of the most popular, oldest known, and widely used medicinal plants in South Africa (Watt and Breyer-Brandwijk, 1962; Dube, 2006; Van Wyk *et al.*, 2009). It is known by many different names, due to the wide use throughout various ethnic groups (Watt and Breyer-Brandwijk, 1932) and is used in the treatment of numerous ailments. This plant is very often grown in home gardens and used for its medicinal properties. The plant is also sold as cuttings in some nurseries in South Africa. The first commercial plantation of the plant occurred in Gouda, in the Western Cape of South Africa in 1995 (Van Wyk, 2011). The fresh leaves of the plant are sold, but more commonly, a tea infusion made from the leaves is sold

as “wildeals” or “wild wormwood” (Van Wyk *et al.*, 2009). The first low-thujone content product came onto the market in 1996 as a tincture and was under the brand of Healer’s Choice (Van Wyk, 2011).

Appendix F.4.

Aspalathus linearis (Burm. F.) Dahlg.

**Family name**

Fabaceae

Vernacular names

rooibos tea (English)

rooibostee (Afrikaans)

Figure F.4.1. The flower of *A. linearis* [Photograph by Prof. A.M. Viljoen (TUT)].

Botanical description

It is a shrub that grows up to two metres in height. The leaves are bright green and are needle-shaped. The flowers are small, yellow and pea-shaped (Van Wyk *et al.*, 2009).

Geographical distribution

The plant is endemic to the Western Cape, particularly the western parts of the province, such as the Cape Peninsula and the Cederburg area (Joubert *et al.*, 2008; Van Wyk *et al.*, 2009).

Traditional medicinal use

It is used as a milk substitute for infants suffering from colic and a health beverage in adults for its' anti-oxidant and anti-ageing activity (Van Wyk *et al.*, 2009). It is also commonly used for GI complaints (antispasmodic) and dermatological problems, such as eczema (Joubert *et al.*, 2008). African women drink the tea during pregnancy to reduce heartburn and nausea and also for its iron content. The plant can also reduce cholesterol levels, and is therefore of benefit in heart disease (Van Wyk *et al.*, 1997; Marnewick *et al.*, 2011).

Plant parts used

Leaves and twigs (Van Wyk *et al.*, 2009).

Dosage form and route of administration

It is consumed orally as a tea (leaves and twigs boiled in water), sometimes together with sugar and milk (Van Wyk *et al.*, 2009). It can also sometimes be applied topically as a cosmetic or manufactured cream (Van Wyk, 2011). Products available on the market include Goudkop rooibos tea from Phytogreen and rooibos tea from Skimmelberg (Figure F.4.2).



Figure F.4.2. Commercial products containing *A. linearis*. A = Rooibos tea (Phytogreen, 2011); B = Rooibos tea (Skimmelberg, 2011b).

Adverse reactions or toxic effects

No adverse effects were found when six cups of rooibos were consumed per day for six weeks (Marnewick *et al.*, 2011). An aqueous extract of fermented and unfermented *A. linearis* was tested on rats over a 10 week period, and no toxic effects were noted against the kidney and liver (Marnewick *et al.*, 2003).

Antimicrobial activity

Aspalathus linearis has been found to have anti-spore and antiviral activity, including anti-HIV activity. The plant also shows activity against *E. coli*, *S. aureus*, *Listeria monocytogenes*, *Streptococcus mutans* and *Saccharomyces cerevisiae* (Schepers, 2001; Almajano *et al.*, 2008; Coetzee *et al.*, 2008).

History and commercialisation of the plant

Aspalathus linearis is a beverage that was discovered by the Khoi-descended people in the Cape and has become a popular health beverage, enjoyed throughout the world. It has been one of South Africa's most successful plants for commercialisation. Rooibos is currently

produced in volumes of 20,000 tonnes per year. It was first commercialised by Benjamin Ginsberg in 1904 and was first marketed as Eleven O’Clock tea (Joubert *et al.*, 2008). Its’ medicinal properties were only later discovered. The red type or rocklands type is the only one that is cultivated for commercial purposes. The plant is harvested, fermented and sold as the well-known “rooibos tea”. It is now also commonly used in the preparation of iced teas (Van Wyk *et al.*, 2009). The tea has a large international market, including Germany, Japan, the United States of America and the Netherlands (Joubert *et al.*, 2008); with the international market far outgrowing the local market (Van Wyk, 2011).

Appendix F.5.

Lippia javanica (Burm. F.) Spreng.



Family name

Verbenaceae

Vernacular names

fever tea, lemon bush (English)

koorsbossie (Afrikaans)

musukudu, bokhukhwane (Tswana)

inzinziniba (Xhosa)

umsuzwane (Zulu)

mumara (Shona)

Figure F.5.1. *Lippia javanica* [Photograph by Prof. A.M., Viljoen (TUT)].

Botanical description

It is a woody shrub standing up to two metres in height. The leaves are hairy and very aromatic, possessing a lemon scent. Flowers appear in dense round clusters and are yellowish in colour (Van Wyk *et al.*, 2009).

Geographical distribution

The plant is found throughout South Africa, more commonly along the eastern and northern parts. It is also found in the northern tropical parts of Africa (Van Wyk *et al.*, 2009).

Traditional medicinal use

Respiratory infections (coughs, colds, bronchitis, influenza), skin infections, GI complaints (dysentery and diarrhoea), sore eyes, fever, malaria, measles, rashes, scabies, lice, skin infections, food poisoning, intestinal worms and disinfecting anthrax-infected meat (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996; Van Wyk *et al.*, 1997; Van Wyk *et al.*, 2009; Van Wyk, 2011).

Plant parts used

Leaves, twigs and sometimes roots (Van Wyk *et al.*, 2009).

Dosage form and route of administration

Weak infusions prepared from leaves, twigs and roots made with milk or water (Van Wyk *et al.*, 2009). Leaves of the plant are also burnt and the smoke inhaled for respiratory complaints. A poultice is also sometimes prepared from the leaves for direct topical application (Watt and Breyer-Brandwijk, 1962). Products available on the market include fever-tree fly and mosquito repellent from CSIR and an essential oil blend from Floracopeia (Figure F.5.2).



Figure F.5.2. Commercial products containing *L. javanica*. A = *L. javanica* fly and mosquito repellent (CSIR, 2012); B = Essential oil blend containing *L. javanica* (Floracopeia, 2012).

Adverse reactions or toxic effects

Toxicity of the *Lippia* species is due to the presence of icterogenins, which very often causes animal poisoning. Photosensitivity reactions have also been noted (Van Wyk *et al.*, 2009). It was acknowledged that some anecdotal evidence suggests that *L. javanica* has a very low level of toxicity in mammals (Madzimure *et al.*, 2011). However, in the study by Madzimure *et al.* (2011) it was found that high doses of *L. javanica* could have adverse implications on the health of humans.

Aqueous extracts of *L. javanica* were tested in mice and within 48 hours of administration, 12.5 – 37.5% of mice became lethargic and 37.5% of the mice died. The post-mortem revealed organ haemorrhage and congestion. It was stated that this could have been due to the

high concentration of xanthine and that this compound is found in many other plant extracts consumed by humans (Madzimore *et al.*, 2011).

Antimicrobial activity

Lippia javanica has shown activity against *S. aureus*, *P. aeruginosa*, *C. albicans* and *C. neoformans* (Huffman *et al.*, 2002). MIC values of 1.6 – 32 mg/ml have been found when the plant was tested against *S. aureus*, *S. epidermidis*, *B. cereus*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *K. pneumoniae*, *C. albicans* and *C. neoformans* (Van Vuuren and Viljoen, 2006).

History and commercialisation of the plant

Lippia javanica is well-known for its medicinal properties in African traditional healing. It is also a very popular plant among avid herbalists and herb gardeners (Le Roux, 2004). The wild harvested leaves have been used since the 1990s in the preparation of herbal teas (Van Wyk, 2011). *Lippia javanica* has not enjoyed as much commercial success as some other southern African medicinal plants, as it is more commonly prepared as a herbal tea from fresh leaves that have been picked from the garden (Van Wyk *et al.*, 2009).

Appendix F.6.

Pelargonium sidoides DC.

**Family name**

Geraniaceae

Vernacular names

silverleaf geranium (English)

kalwerbossie (Afrikaans)

umckaloabo (Zulu)



Figure F.6.1. The flower of *P. sidoides* (<http://nprcdb.com/blog/?p=106>) and the roots of *P. sidoides* [Photograph by Prof. A.M. Viljoen (TUT)].

Botanical description

It is a short plant with crowded leaves that are heart-shaped and velvety. Flowers are reddish-purple, almost black in colour (Lawrence, 2001).

Geographical distribution

The plant is found throughout the Eastern Cape, Lesotho, Free State and southern parts of Gauteng (Lawrence, 2001).

Traditional medicinal use

Respiratory infections (bronchitis, sinusitis, influenza, pneumonia, pharyngitis, common cold, tonsillitis), STI's, GI complaints (diarrhoea and dysentery), wounds and worm infestations in cattle (Watt and Breyer-Brandwijk, 1996; Hutchings *et al.*, 1996; Brendler and Van Wyk,

2008; Van Wyk *et al.*, 2009; Kolodziej, 2011, Van Wyk, 2011). The crushed roots are mixed in water and given to infants for an upset stomach (Matsiliza and Barker, 2001).

Plant parts used

Roots (tubers) (Van Wyk *et al.*, 2009).

Dosage form and route of administration

Aqueous root decoctions or infusions made with milk or water are orally administered, but can also be used as a topical preparation. Roots can be chewed or powdered for ingestion with food (Watt and Breyer-Brandwijk, 1996). Products available on the market include Umcka cough mixture from Nature's Way and drops from Medico Herbs (Figure F.6.2).



Figure F.6.2. Commercial products containing *P. sidoides*. A = Umcka cough mixture (Nature's Way Products, 2008); B = *Pelargonium sidoides* drops (Medico Herbs, 2009d).

Adverse reactions or toxic effects

Some common adverse effects include GI disorders, central nervous system (CNS) disorders, ear and labyrinth disorders and at very high concentrations, liver toxicity due to coumarin and tannin content (Carmela *et al.*, 2012). Eighteen clinical trials have been conducted, with most being randomized, double-blind and placebo controlled. It was found that there was an overall safety and very low adverse effect profile (Brendler and Van Wyk, 2008). There were fifteen reports that *P. sidoides* had caused hepatotoxicity, however, these claims were queried by authors Carmela *et al.* (2012) and Teschke *et al.* (2012).

Antimicrobial activity

Pelargonium sidoides has shown activity against *Mycobacterium tuberculosis* (Mukinda and Syce, 2007). The organic extracts of the plant also show activity against *S. aureus*, *Streptococcus pneumoniae*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Haemophilus influenzae*, with MIC values ranging from 600 – 7,500 µg/ml (Kolodziej, 2011).

History and commercialisation of the plant

Pelargonium sidoides was first commercialised in 1898, after which the plant has experienced great commercial success throughout the world (Brendler and Van Wyk, 2008). *Pelargonium sidoides* has been central in the treatment in GI and respiratory illnesses for decades in a large population of southern Africa (Kolodziej, 2011). It is a highly valued plant in traditional healing practices, not only for its' curative properties, but for its' palliative effects too (Kolodziej, 2011). The plant was initially marketed as a treatment in tuberculosis infections. Currently, the plant is mainly used in the treatment of the respiratory infection, bronchitis, as well as for GI complaints. It has been marketed as a tincture of the roots (Van Wyk, 2011). The plant has not only gained popularity in southern Africa, but in European countries too (Kolodziej, 2011).

Appendix F.7.

Sutherlandia frutescens (L.) R. Br.

**Family name**

Fabaceae

Vernacular names

cancer bush (English)

kankerbos (Afrikaans)

Figure F.7.1. *Sutherlandia frutescens* [Photograph by Prof. S.F. Van Vuuren (WITS)].

Botanical description

It is a small shrub growing up to a metre in height. The leaves are hairy, with a silvery appearance. Flowers are large and bright red in colour (Van Wyk *et al.*, 2009).

Geographical distribution

The plant only occurs naturally in the southern parts of Africa (Van Wyk *et al.*, 2009).

Traditional medicinal use

General health tonic and immunity booster (Crouch *et al.*, 2006), respiratory infections (chronic bronchitis, colds, influenza), UTI's, wounds, eye infections, GI complaints, gynaecological problems, rheumatism, backache, fever, oedema, diabetes, stress, internal cancer and septicaemia (Watt and Breyer-Brandwijk, 1962; Drewes *et al.*, 2006; Van Wyk and Albrecht, 2008; Van Wyk *et al.*, 2009; Van Wyk, 2011).

Plant parts used

Leaves (Van Wyk *et al.*, 2009).

Dosage form and route of administration

Strong decoctions or alcoholic tinctures made from leaves are used internally as well as externally (Van Wyk *et al.*, 2009). Aqueous infusions are also prepared and used as an antiseptic wash, eye wash or douche. Whole fruit are also sometimes consumed for GI complaints (Scott and Springfield, 2004b). Products available on the market include cancer bush capsules and tea from Medico Herbs, Western Cape (Figure F.7.2).



Figure F.7.2. Commercial products containing *S. frutescens*. A = Cancer bush capsules (Medico Herbs, 2009e); B = Cancer bush tea bags (Medico Herbs, 2009f).

Adverse reactions or toxic effects

The long history of traditional use, with no reports on any serious adverse effects has led to the belief that *S. frutescens* is generally safe (Van Wyk and Albrecht, 2008). The South African Ministry of Health stated that *S. frutescens* was safe to use, based on primate safety studies (Mills *et al.*, 2005). Excessive amounts of plant consumption can cause emesis. Moderate use can result in sweating and mild purgation (Scott and Springfield, 2004b). No cytotoxicity was observed when *S. frutescens* ethanolic extracts were tested against CA-9KB cell lines at a concentration of 20 µg/ml (Charlson, 1980). When *S. frutescens* aqueous extract was tested at 100 µg/ml for cytotoxicity against three human cancer cell lines, namely DU-145 (prostate cancer cells), MDA-MB-231 (breast cancer cells) and MCF-7 (breast cancer cells), no pronounced cytotoxicity was exhibited (Steenkamp and Gouws, 2006).

Antimicrobial activity

Sutherlandia frutescens hexane extract shows activity against *S. aureus* (MIC of 300 µg/ml), *E. faecalis* (1.25 mg/ml) and *E. coli* (2.50 mg/ml) (Katerere and Eloff, 2005). Activity also extends to other staphylococcal species. Anti-HIV activity has also been documented (Fu *et al.*, 2008). No activity has been seen against *P. aeruginosa* and *C. albicans* (Scott and Springfield, 2004b).

History and commercialisation of the plant

Sutherlandia frutescens is a very old Cape remedy. The medicinal properties were initially discovered by the Khoi and Nama people (Van Wyk *et al.*, 2009). It has been used by the Khoi-San and Cape Dutch people since 1895 for internal cancers (Van Wyk and Albrecht, 2008). Plantations were initially established in the late 1990s (Van Wyk, 2011), where only small-scale cultivation and commercialisation took place in the Cape Province (Drewes, 2012). The first branded products available were tablets prepared from powdered leaves. Phyto Nova (Pty) was responsible for the large-scale cultivation of *S. frutescens* (Van Wyk, 2011; Drewes, 2012). The plant is usually sold commercially as dried leaves or as a strong tincture, known as “cancer bush” or “kankerbos” (Van Wyk *et al.*, 2009) and has recently gained popularity due to its’ beneficial effects seen in HIV/AIDS patients in South Africa (Katerere and Eloff, 2005).

Appendix G

Antimicrobial agents investigated in this study

Appendix G.1.

Ciprofloxacin

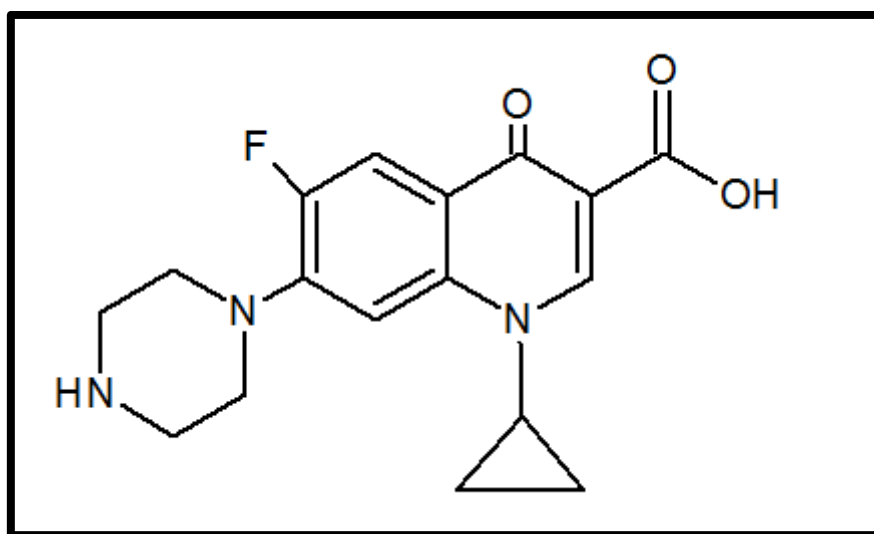


Figure G.1. Ciprofloxacin chemical structure (Katzung *et al.*, 2009).

Class of antimicrobial

Fluoroquinolone (Katzung *et al.*, 2009; SAMF, 2012).

Mechanism of action

The antibiotic inhibits DNA gyrase, thereby interfering with DNA reproduction of the microbe (Merck Manual, 2006).

Medicinal uses

UTI's, respiratory tract infections, GIT infections, STI's, skin, bone and soft tissue infections. It is also the drug of choice for typhoid fever (SAMF, 2012).

Dosage form and route of administration

Ciprofloxacin can be administered orally, intravenously (IV), parenterally or topically in eye preparations (Merck Manual, 2006).

Products available on the market

Ciprobay[®] tablets or IV formulations by Bayer Schering. Aspen Ciprofloxacin[®] tablets, Cipro-Hexal[®] tablets by Arrow Pharma, Cliploxx[®] tablets by Cipla Medpro, Cifloc[®] tablets by Dr Reddy's and Sabax Ciprofloxacin[®] IV from Specpharm (SAMF, 2012).

Adverse reactions or toxic effects

Ciprofloxacin rarely demonstrates toxic effects, however, the most common effects include GI effects, CNS toxicity, along with damaging effects on cartilage, and cardiovascular system (CVS) toxicity, via the prolonging of the QT interval (Merck Manual, 2006). It can also cause damage to cartilage in the foetus and should be avoided in pregnancy (SAMF, 2012).

Antimicrobial spectrum

It is highly active against Gram-negative micro-organisms, particularly against *Pseudomonas aeruginosa*, *Enterobacteriaceae*, *Haemophilus* species, *Legionella* and *Neisseria* species (Merck Manual, 2006), with moderate to good activity against Gram-positive bacteria (Katzung *et al.*, 2009).

Resistance toward antimicrobial

In the 1980s, resistance of microbes toward fluoroquinolones increased rapidly. It was estimated that in a New York City tertiary hospital, that more than 80% of MRSA strains were resistant toward the fluoroquinolones (Swartz, 1994). Resistance to this antibiotic has developed among the *Gonococcus* species, rendering this antibiotic ineffective in the treatment of gonorrhoea (SAMF, 2012). There has also been the emergence of fluoroquinolone-resistant *S. aureus*, where the microbe has developed a reduced sensitivity of the DNA gyrase toward the antibiotic or via a reduced permeability of the microbe toward ciprofloxacin. Another mechanism by which the bacteria develop resistance toward ciprofloxacin is via an increased efflux of the antibiotic. Point mutations in the quinolone binding areas result in high-level resistance (Merck Manual, 2006; Katzung *et al.*, 2009).

Appendix G.2.

Erythromycin

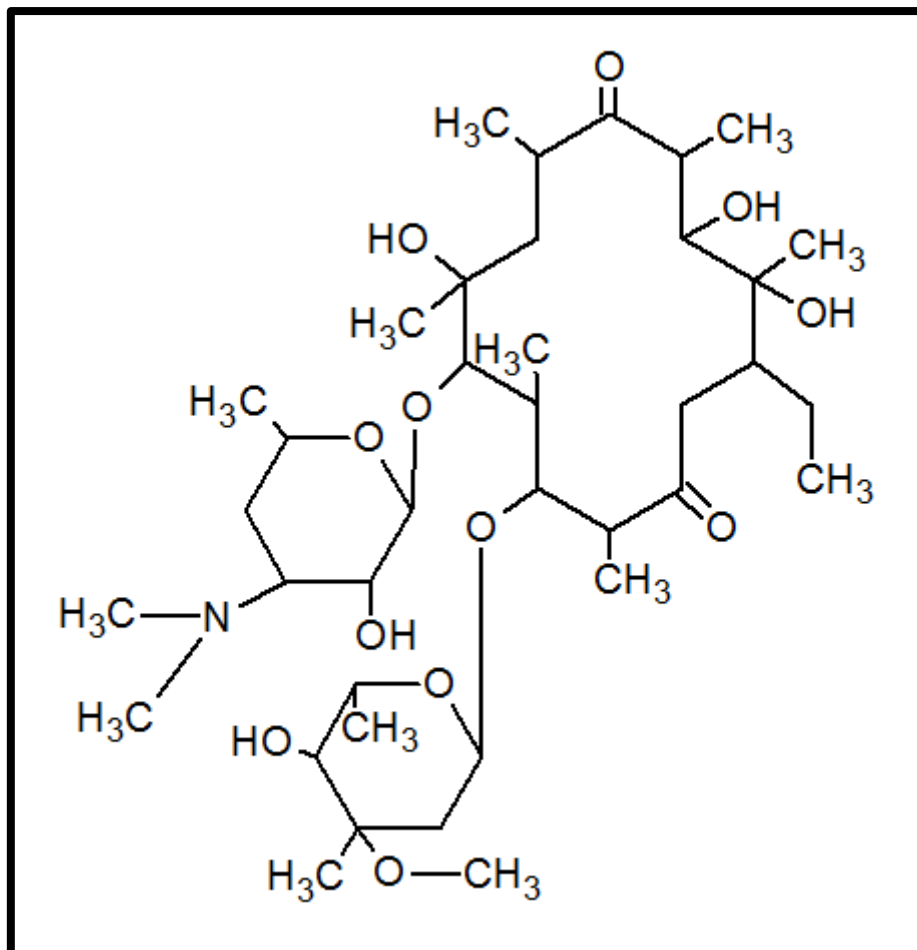


Figure G.2. Erythromycin chemical structure (Katzung *et al.*, 2009).

Class of antimicrobial

Macrolide (Katzung *et al.*, 2009; SAMF, 2012).

Mechanism of action

The antibiotic inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit (Merck Manual, 2006).

Medicinal uses

Sexually transmitted infections, respiratory tract infections caused by Gram-positive bacteria, including whooping cough and pneumonia caused by *Legionella* species. It is also used as an

alternative to penicillin, in the treatment of Gram-positive infections, where a penicillin allergy exists (Merck Manual, 2006; SAMF, 2012).

Dosage form and route of administration

The antibiotic can be administered orally, parenterally or IV (Merck Manual, 2006; SAMF, 2012).

Products available on the market

Purmycin[®] capsules and suspension by Aspen Pharmacare, Adco-Erythromycin[®] capsules or suspension, Betamycin[®] suspension from Ranbaxy, Erymycin[®] suspension or capsules by Aspen Pharmacare and lastly Erythrocin[®] IV from Pharmaco (SAMF, 2012).

Adverse reactions or toxic effects

Erythromycin can show GI effects, CVS toxicity, along with hepatotoxicity and auditory toxicity (Merck Manual, 2006; Katzung *et al.*, 2009).

Antimicrobial spectrum

It is active against Gram-positive microbes, particularly pneumococci, streptococci, staphylococci and corynebacteria (Katzung *et al.*, 2009). It has some anaerobic activity and has a very limited Gram-negative activity. It is active against *Bordetella pertussis*, *Legionella* species, *Chlamydia* species, *Campylobacter* species, *Rickettsia* species, as well as some *Mycobacteria* species (Merck Manual, 2006; Katzung *et al.*, 2009).

Resistance toward antimicrobial

Resistance toward this agent has become common among streptococcal species (SAMF, 2012). Resistance has also been seen among staphylococcal species, where for example, *S. aureus* is capable of developing resistance via increased antibiotic efflux activity (Merck Manual, 2006). Resistance has been found to be mainly plasmid-mediated and also inducible, where the binding site for the antibiotic is altered (Swartz, 1994; Katzung *et al.*, 2009).

Appendix G.3.

Gentamicin

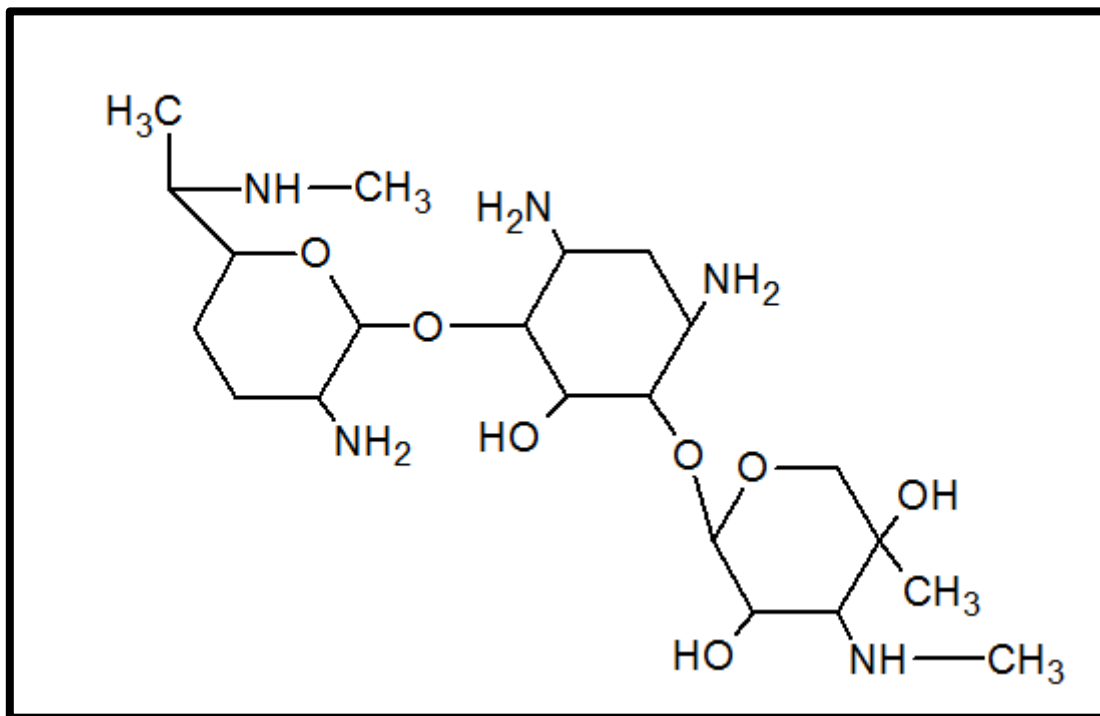


Figure G.3. Gentamicin chemical structure (Katzung *et al.*, 2009).

Class of antimicrobial

Aminoglycoside (Katzung *et al.*, 2009; SAMF, 2012).

Mechanism of action

The antibiotic inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit (Merck Manual, 2012).

Medicinal uses

It is used for the treatment of serious Gram-negative infections, such as sepsis and pneumonia. It is mainly used in combination with another antimicrobial agent (Katzung *et al.*, 2009).

Dosage form and route of administration

Aminoglycosides are usually given IV at a very slow rate. These agents can also be injected directly into the eye or brain (Merck Manual, 2006). Gentamicin has also been used intramuscularly and topically for wounds and eye infections (Katzung *et al.*, 2009).

Products available on the market

Garamycin[®] for injection by Schering-Plough, Aspen Gentamicin[®] and Sandoz Gentamicin[®] for injection, Gentamycin-Fresenius[®] for injection by Fresenius Karbi and Sabax Gentamix[®] IV by AI Critical Care (SAMF, 2012).

Adverse reactions or toxic effects

All aminoglycosides produce renal toxicity, which is usually reversible, and ototoxicity which can often be irreversible. The risk for toxicity is increased with larger doses, longer durations of therapy, more frequent dosing, all resulting in higher blood levels (Merck Manual, 2006). Aminoglycosides can also cross the placenta and cause toxicity in the foetus, therefore should be avoided during pregnancy. CNS toxicity and blood component toxicity has also been noted (SAMF, 2012).

Antimicrobial spectrum

This agent is active against most Gram-negative aerobic micro-organisms and is therefore very often first line treatment for serious Gram-negative infections, especially those caused by *P. aeruginosa*. It is, however, ineffective against anaerobes (Merck Manual, 2006). It is active against some Gram-positive microbes, which is limited to strains of staphylococci (Katzung *et al.*, 2009).

Resistance toward antimicrobial

Resistance of nosocomial infections toward gentamicin has become common, since it has been used in many hospitals and clinics as the standard aminoglycoside (SAMF, 2012). Some Gram-negative *Bacilli* species and methicillin-resistant staphylococci have shown resistance. Resistance of the *Enterococcus* species toward gentamicin has also resulted, due to the production of inactivating enzymes (Merck Manual, 2006).

Appendix G.4.

Penicillin G (Benzylpenicillin)

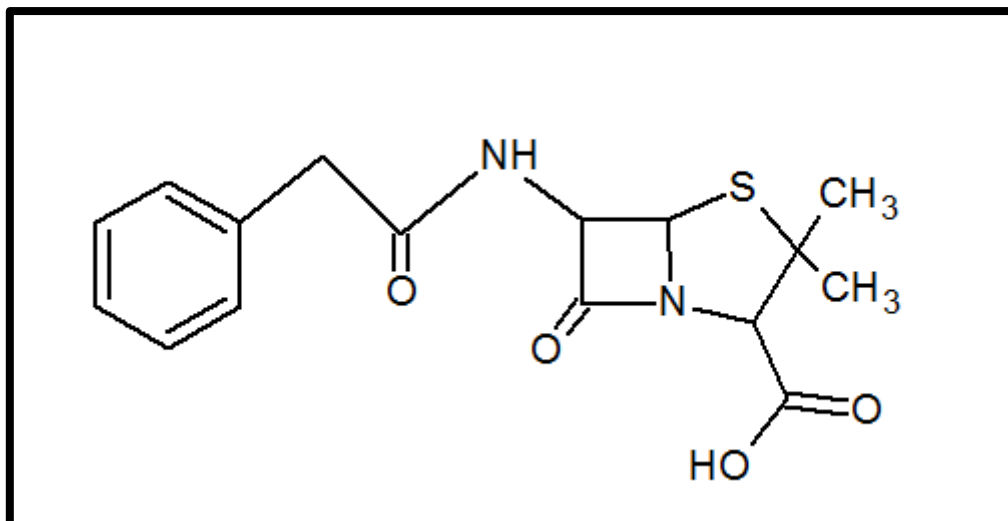


Figure G.4. Penicillin G potassium chemical structure (Katzung *et al.*, 2009).

Class of antimicrobial

Beta-lactam (Katzung *et al.*, 2009; SAMF, 2012).

Mechanism of action

The antibiotic inhibits bacterial cell wall synthesis (SAMF, 2012).

Medicinal use

It is used in the treatment of streptococcal tonsillitis or pharyngitis and other respiratory tract infections, endocarditis and meningitis, caused by sensitive micro-organisms, syphilis and gangrene (Merck Manual, 2006; SAMF, 2012).

Dosage form and route of administration

Benzylpenicillin is administered as a deep intramuscular injection (Merck Manual, 2006) and intravenously (SAMF, 2012).

Products available on the market

Benzyl Penicillin-Fresenius® by Bodene, and Bio-Pen® by Biotech (SAMF, 2012).

Adverse reactions or toxic effects

Penicillin most commonly causes hypersensitivity effects, including anaphylaxis. The antibiotic causes CNS toxicity at high doses and all penicillins cause nephritis and have some toxic effects on the blood components (Merck Manual, 2006).

Antimicrobial spectrum

Penicillin has a very narrow-spectrum of activity, which is mainly limited to Gram-positive micro-organisms. It is still the first choice for the treatment of Gram-positive infections, as well as anaerobes. Enterococci are less susceptible to penicillin G, thus a combination with an aminoglycoside is often needed to enhance its efficacy, for example, in the treatment of enterococcal endocarditis (SAMF, 2012). It is also active against Gram-negative cocci, however, it is less active against Gram-negative rods (Katzung *et al.*, 2009).

Resistance toward antimicrobial

Resistance to penicillin is a common occurrence. Resistance is due to either the inactivation of the antibiotic by the β -lactamases produced by the microbes, or by modification of the target site, impaired penetration of the drug or via the increased efflux of the antibiotic (Katzung *et al.*, 2009; Merck Manual, 2006). Around 90 – 95% of *S. aureus* strains have been found to be resistant toward penicillin (Hemaiswarya *et al.*, 2008).

Appendix G.5.

Tetracycline

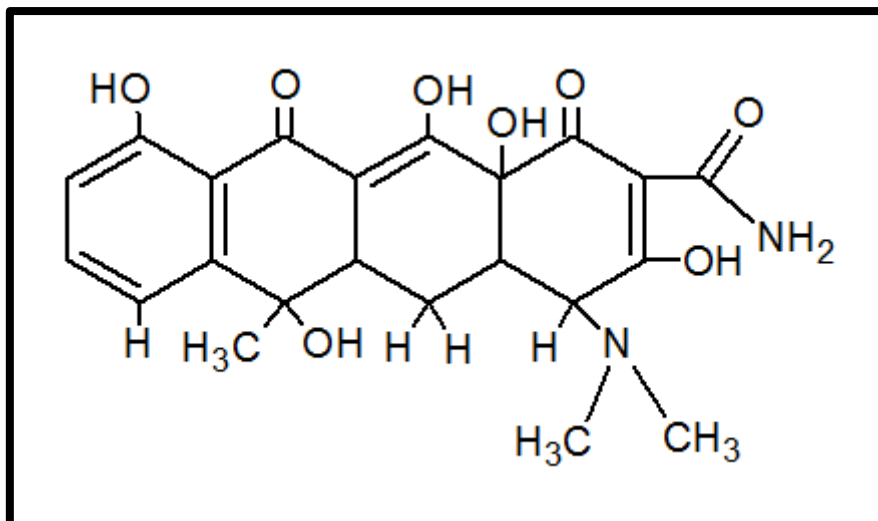


Figure G.5. Tetracycline chemical structure (Katzung *et al.*, 2009).

Class of antimicrobial

Tetracycline (Katzung *et al.*, 2009; SAMF, 2012).

Mechanism of action

The antibiotic is a bacterial protein synthesis inhibitor and binds to the 30S ribosomal subunit (Merck Manual, 2006).

Medicinal use

Tetracycline is used in the treatment of *Rickettsia* and *Chlamydia* infections and also in chronic bronchitis, acne and infections caused by spirochetes (SAMF, 2012). It is used in combination regimens against *Helicobacter pylori*. It is also active against bacterial *Vibrio*-related infections (Katzung *et al.*, 2009).

Dosage form and route of administration

Tetracyclines are orally administered (Merck Manual, 2006).

Products available on the market

There are no products available in South Africa containing tetracycline, but only products containing its derivatives. Elsewhere though, Hostacycline[®] capsules by Aventis and Tetracycline HCL[®] capsules by IVAX are available.

Adverse reactions or toxic effects

Tetracyclines can demonstrate hepatotoxicity, GI effects, photosensitivity and damaging effects on bone and teeth in children (Merck Manual, 2006). Renal toxicity can occur with the use of out-dated tetracycline preparations (Katzung *et al.*, 2009).

Antimicrobial spectrum

Tetracyclines have a broad-spectrum activity, with activity ranging from Gram-positive and Gram-negative micro-organisms, to atypical pathogens and parasites (Chopra and Roberts, 2001). It is first line therapy for *Chlamydia* infections, and is also used in the treatment of *Propionibacterium acnes*, rickettsiae, *H. pylori* and *Vibrio cholerae* (Katzung *et al.*, 2009).

Resistance toward antimicrobial

Tetracyclines were used extensively, due to their low adverse effects and desirable antimicrobial activity. However, their extensive use has led to development of resistance. There has been increased resistance toward this agent, for example, *S. aureus* has been found to develop resistance toward tetracycline via increased efflux pump activity (Merck Manual, 2006). The identification of the resistance mechanism allowed for the development of newer tetracyclines to which no resistance has developed. Tetracyclines have also been combined with agents which demonstrate an inhibitory effect toward the resistance mechanisms of micro-organisms, which has rendered the old tetracyclines active once more (Chopra and Roberts, 2001). Resistance of staphylococci toward tetracyclines has been attributed to the mechanism by which microbes are able to synthesise new membrane protein, which then prevents the accumulation of the antibiotic in the microbial cell (Swartz, 1994). Katzung *et al.* (2009) describes three mechanisms by which tetracyclines develop resistance, which includes increased efflux or impaired influx, ribosome protection due to proteins interfering with binding to the ribosomes and lastly enzymatic inactivation of the antibiotic.

Appendix G.6.

Amphotericin B

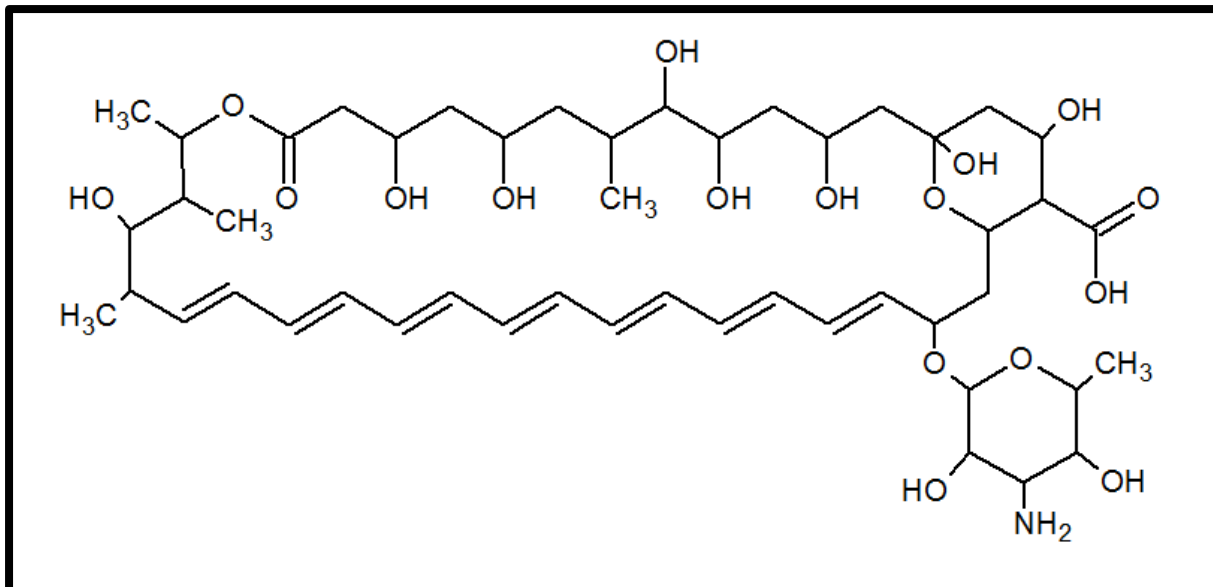


Figure G.6. Amphotericin B chemical structure (Katzung *et al.*, 2009).

Class of antimicrobial

Polyene antifungal (Katzung *et al.*, 2009; SAMF, 2012).

Mechanism of action

The antifungal binds to sterols, such as ergosterol, in the cell membrane of the fungal species and affects the fungal cell membrane integrity (SAMF, 2012).

Medicinal use

It is used for treating opportunistic micro-organisms such as mycoses which has spread systemically, for example, candidiasis, cryptococcosis and histoplasmosis (SAMF, 2012). It can be used in the treatment of most fungal infections (Merck Manual, 2006).

Dosage form and route of administration

Amphotericin B is administered intravenously or sometimes even intrathecal therapy for meningitis (Merck Manual, 2006). Local and topical administration has also shown success for the treatment of eye infections (Katzung *et al.*, 2009).

Products available on the market

Fungizone[®] IV from BM Squibb and Ambisone[®] IV from Key Oncologics (SAMF, 2012).

Adverse reactions or toxic effects

Amphotericin B has a very high level of toxicity, however, it is still the standard treatment for severe systemic mycoses. Renal toxicity is the most common toxic effect and renal functioning needs to be monitored throughout treatment. GI effects, hepatotoxicity, CVS toxicity and auditory and visual effects have also been noted (Merck Manual, 2006). Infusion-related reactions include headache, vomiting, fever, chills, muscle spasms and hypotension (Katzung *et al.*, 2009).

Antimicrobial spectrum

Cryptococcus species, *Candida* species, *Histoplasma capsulatum*, *Aspergillus* species, *Blastomyces dermatitidis*. It is also used for sporotrichosis and leishmaniasis (Merck Manual, 2006; Katzung *et al.*, 2009).

Resistance toward antimicrobial

Resistance toward amphotericin B is rare; however, an increase in resistance has occurred recently. This may be due to the large proportion of the population being immunocompromised, mainly due to the prevalence of HIV/AIDS, but also due to chemotherapy. The resistance has been reported against *Candida* and *Cryptococcus* species (O'Shaughnessy *et al.*, 2009). The mechanism of resistance toward amphotericin B is mainly via the alteration of membrane lipids, particularly ergosterol, of the fungal species (Vanden Bossche *et al.*, 1998). The result of impaired ergosterol binding can be due to a decreased membrane concentration of ergosterol or via the modification of the sterol target causing a reduced affinity for amphotericin B (Katzung *et al.*, 2009).

Appendix G.7.

Nystatin

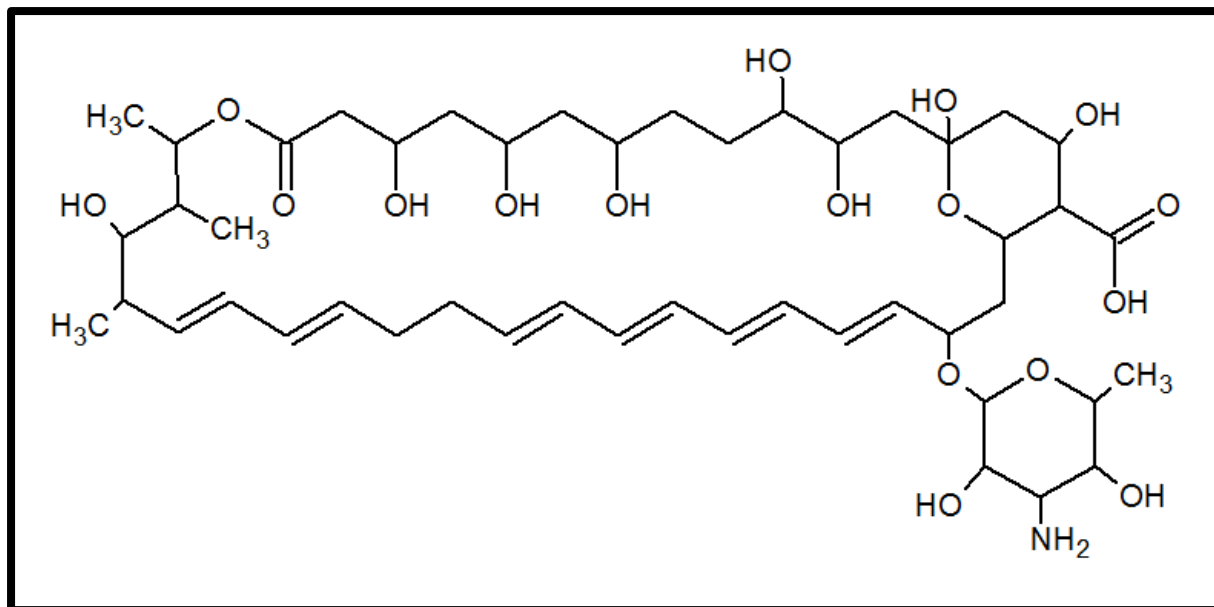


Figure G.7. Nystatin chemical structure (Katzung *et al.*, 2009).

Class of antimicrobial

Polyene antifungal (Katzung *et al.*, 2009; SAMF, 2012).

Mechanism of action

The antifungal binds to sterols, such as ergosterol, in the cell membrane of the fungal species and affects the fungal cell membrane integrity (SAMF, 2012).

Medicinal use

It is only used for cutaneous and mucocutaneous candidal infections and is not effective for systemic infections or infections caused by dermatophytes (Merck Manual, 2006).

Dosage form and route of administration

Nystatin can be administered vaginally and also has a mouth rinse, however, cannot be administered IV due to the toxicity profile (Katzung *et al.*, 2009; SAMF, 2012). Suspensions can also be orally ingested for GI infections, however, is often limited due to the unpleasant taste (Katzung *et al.*, 2009).

Products available on the market

Canstat[®] cream or vaginal tablets from Aspen Pharmacare, Nystacid[®] ointment or suspension from Aspen Pharmacare, Mycostatin[®] suspension from BM Squibb and Candacide[®] from Ranbaxy (SAMF, 2012).

Adverse reactions or toxic effects

Nystatin can cause GI effects, skin rashes, facial swelling, myalgia and bronchospasm. Some cardiotoxicity has also been noted (Merck Manual, 2006). It cannot be used for parenteral administration due to its toxicity. Little toxicity would occur with oral ingestion due to the minimal absorption from the GIT (Katzung *et al.*, 2009).

Antimicrobial spectrum

It is active against most *Candida* species (Katzung *et al.*, 2009).

Resistance toward antimicrobial

Resistance toward nystatin has also only recently developed, similarly as seen with amphotericin B. The mechanism of action, as with amphotericin B, is related to the alteration of the membrane lipids, such as ergosterol, which is the binding site for polyenes (Dick *et al.*, 1980).

Appendix H

Ethics clearance certificate for microbial cultures

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

University
of the Witwatersrand,
Johannesburg



Ref: W-C.I-130726-1

26/07/2013

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Prof S van Vuuren, Ms Z Hubsch (student no 300896).

Project title: Antimicrobial efficacy testing and toxicity profiles of conventional antimicrobial agents in combination with commercially relevant Southern African medicinal plants.

Reason: This is a laboratory study using commercial bacterial and fungal cultures including *S. aureus*, *B. cereus*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *C. albicans*, *C. neoformans*. No humans are involved.



Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Zanele Ndlovu, Wits Research Office

Appendix I

Ethics clearance certificate for HEK-293 cell line

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical))

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

University
of the Witwatersrand,
Johannesburg

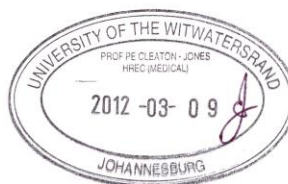


Ref: W-CJ-120309-3

09/03/2012

TO WHOM IT MAY CONCERN:

- Waiver:** This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).
- Investigator:** Prof RL van Zyl, Z Hubsch (student no 300896)
- Project title:** Antimicrobial efficacy testing and toxicity profiles of conventional antimicrobial agents in combination with commercially relevant Southern African medicinal plants.
- Reason:** Commercial cell lines will be used - colorectal carcinoma (Caco-2), hepatocarcinoma (HepG2) and human kidney epithelial (Graham). No humans are involved.



Professor Peter Cleaton-Jones
Chair: Human Research Ethics Committee (Medical)

copy: Anisa Keshav, Research Office, Senate House, Wits

Appendix J

Chemotherapeutic agents in toxicity studies

Appendix J.1.

Quinine

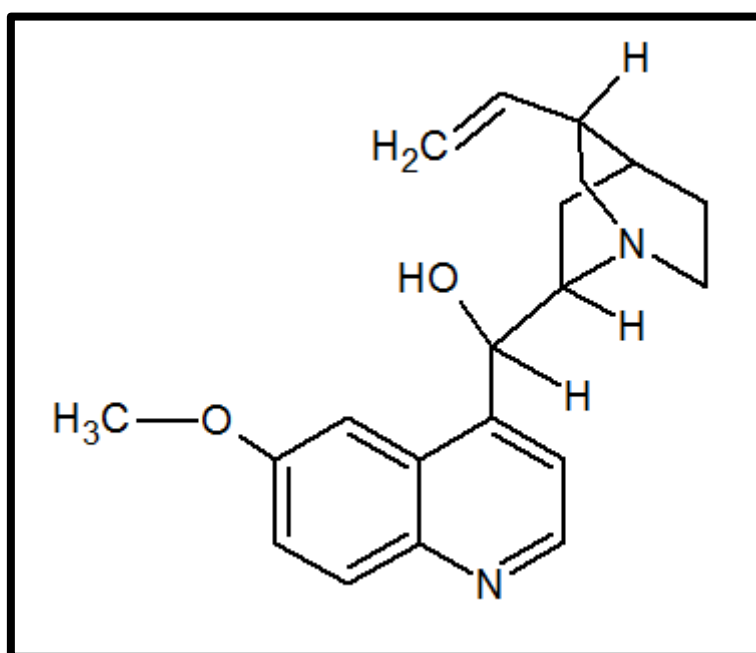


Figure J.1. Chemical structure of quinine (Katzung *et al.*, 2009).

Class of drug

Antiprotozoal (antimalarial) (Katzung *et al.*, 2009).

Mechanism of action

The mechanism of action is unknown, however, it is known to have effective blood schizonticide activity against the four species of malarial parasites affecting humans. It is also gametocidal against *Plasmodium vivax* and *Plasmodium ovale* (Katzung *et al.*, 2009).

Medicinal uses or spectrum of activity

Quinine is active against all four species causing malaria in humans, namely *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *Plasmodium malariae*. It has been used as first-line treatment for *P. falciparum* malaria, and is highly effective in the treatment of severe disease. It is also used in the treatment of babesial infections, particularly infections caused by *Babesia microti* (Katzung *et al.*, 2009).

Dosage form and route of administration

Quinine can be administered orally as tablets for uncomplicated malaria, or as an IV infusion for severe malaria. It is also administered intramuscularly (SAMF, 2012).

Products available on the market

Quinine Dihydrochloride-Fresenius[®] for IV administration and Lennon-Quinine Sulphate[®] tablets by Aspen Pharmacare (SAMF, 2012).

Adverse reactions or toxic effects

Adverse effects that commonly occur at therapeutic dosages, known as cinchonism, include tinnitus, headache, nausea, dizziness, flushing and visual disturbances. Hypersensitivity reactions have also occurred, such as skin rashes, urticarial, angioedema and bronchospasm. Hypoglycaemia is also common with therapeutic doses. Severe hypotension has also resulted from rapid IV infusion of the drug. Toxic haematological effects include haemolysis, leukopenia, agranulocytosis and thrombocytopenia. Quinine can also cause uterine contractions, particularly in the third trimester. Even so, it is still the drug of choice for severe *P. falciparum* malaria in pregnancy. The toxicity related to the quinine therapy has been said to complicate therapy (Katzung *et al.*, 2009). Use is cautioned in renal and hepatic impairment (SAMF, 2012).

Resistance toward the drug

Resistance toward quinine is uncommon in South Africa, however, may be on the increase. In South-east Asia, resistance has developed in many areas (Katzung *et al.*, 2009).

Appendix J.2.

Camptothecin

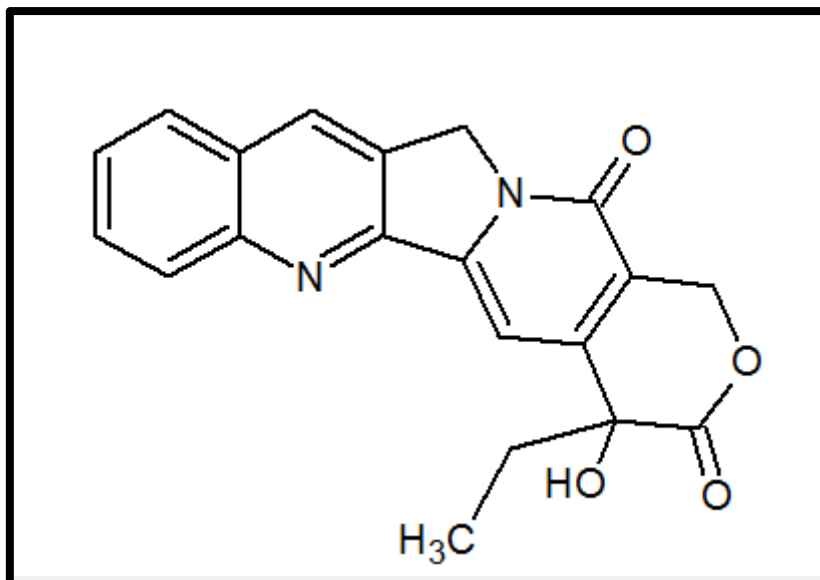


Figure J.2. Chemical structure of camptothecin (Katzung *et al.*, 2009).

Class of drug

Topoisomerase inhibitor (antineoplastic) (SAMF, 2012).

Mechanism of action

It inhibits the activity of topoisomerase I and II, resulting in the damage of DNA which inhibits replication (Merck Manual, 2006; Katzung *et al.*, 2009).

Medicinal use

This agent is used as second-line treatment of advanced ovarian cancer and small cell lung cancer. Its prodrug is used as first-line therapy for metastatic colorectal cancer, when combined with 5-fluoruracil and leucovorin (Katzung *et al.*, 2009).

Dosage forms and route of administration

The drug is only available for IV administration, as either a concentrate or a powder to be reconstituted (SAMF, 2012).

Products available on the market

Campto[®] by Pfizer, Accord-Irinotecan[®] by Mylan and Sandoz Irinotecan[®] are all IV preparations containing irinotecan. Hycamtin[®] by GlaxoSmithKline is a powder of topotecan, which is reconstituted for IV infusion (SAMF, 2012).

Adverse reactions or toxic effects

Myelosuppression and diarrhoea are the common adverse effects associated with this drug. Delayed diarrhoea can be life-threatening, especially in neutropenic patients. Therefore, careful monitoring and hydration is needed (Katzung *et al.*, 2009; SAMF, 2012). Alopecia is also associated with irinotecan use (Merck Manual, 2006).